Structure and Function of Qß Replicase Binding Sites on Qß RNA

INAUGURAL - DISSERTATION

zur

Erlangung der Philosophischen Doktorwürde

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FRANÇOIS MEYER

luxemburgischer Staatsangehöriger

Begutachtet von

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LEIDEN
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à mes chers parents

à Violaine

Herrn Prof. Dr. Charles Weissmann und Herrn Dr. Hans Weber unter deren Leitung diese Arbeit entstanden ist, möchte ich für die wissenschaftliche Förderung, die sie mir zuteil werden liessen meinen allerherzlichsten Dank aussprechen.

Für die Mitteilung bisher unveröffentlichter Resultate, sowie für Präparate von α-^{32}P markierten Nukleosid-Triphosphaten, danke ich Herrn Prof. Dr. Martin Billeter.

Bei allen jetzigen und ehemaligen Mitarbeitern unseres Institutes bedanke ich mich für die hilfreiche Zusammenarbeit und anregende Diskussionen.

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SUMMARY

The interaction between Qβ replicase and Qβ RNA was studied by binding the replicase RNA complexes, after partial RNase T_1 digestion to Millipore filters. The retained RNA fragments were analyzed by one-dimensional polyacrylamide gel electrophoresis or after complete digestion with RNase T_1 by two-dimensional polyacrylamide gel electrophoresis.

Depending on reaction conditions, Qβ replicase is able to bind specifically to either one or both of 2 regions on Qβ RNA. One site (S-site) is identical to the one described by Weber et al, (1972). It is located at the beginning of the coat cistron and is probably responsible for the translational repressor action of Qβ replicase. The second site(M-site) has been characterized as 3 non-overlapping RNA segments located within the replicase cistron, not far from its starting point. The complete nucleotide sequence of the largest M-site fragment (chain length,173 nucleotides) was determined. No significant sequence homology was found between M-site and S-site RNA fragments.
An interaction with the 3'-terminus of Qβ RNA was found only under conditions where initiation of Qβ RNA synthesis occurs.

In a different approach,when replicase was challenged with a complete RNase T_1 digest of Qβ RNA, the enzyme selectively bound T_1 oligonucleotides derived from the M-and S-sites and the 3'-terminal region, but not the T_1 oligonucleotide from the very 3'-end. Structural features on an RNA segment which might be essential for recognition by replicase are discussed.

S-and M-site RNA fragments were selectively bound to replicase as shown by binding competition with unlabeled random Qβ RNA fragments and E. coli ribosomal RNA fragments of the same average size. Both S-and M site RNA were strongly inhibiting initiation of Qβ RNA synthesis. The effect was specific since ribosomal RNA fragments of similar length showed no inhibition.

Evidence is discussed, suggesting that the interaction at the M-site is required for the formation of a productive initiation complex, whereas the binding at the S-site is not essential.

1. INTRODUCTION

1.1. General Biology of Bacteriophage Qβ

The RNA-containing coliphages may be classified into 5 groups, both by serological and biochemical criteria (Miyake et al., 1971; Watanabe et al., 1967; Scott, 1965).

The first group comprises phages such as f2, R17 and MS2, which are quite similar among each other and differ considerably from Qβ, a member of the third group. These four phages have been investigated extensively, whereas phages of groups 2, 4 and 5 have not been studied to the same extent (Stavis & August, 1970; Weissmann et al., 1973; Zinder, 1975). RNA phage particles are roughly spherical with a diameter of about 250 Å (Fischbach et al., 1965; Zipper et al., 1971). The single stranded RNA genome consists of 3500 (group 1) to 4500 (group 2) nucleotides (Sinha et al., 1965; Boedtker, 1971) and has a high degree of secondary and tertiary structure (Boedtker, 1968). It is surrounded by 180 coat protein molecules; in addition, each particle contains one molecule of maturation (A or in Qβ, A2) protein, which is essential for the binding of the phage to its host (Lodish et al., 1965; Roberts & Steitz, 1967; Krahn & Paranchych, 1971). Phage Qβ contains a few molecules of a third virus specific protein, called IIb or A1 (Garwes et al., 1969), which is essential for the formation of a viable particle, for as yet unknown reasons (Hofstetter et al., 1974). Genetic analyses of group 1 and group 3 phages have identified 3 cistrons, one coding for maturation protein, a second for coat protein and a third for the β subunit of the viral RNA polymerase (Gussin, 1966; Horiuchi et al., 1966; Horiuchi & Matsuhashi, 1970). The genetic map of Qβ RNA is shown in Fig. 1.

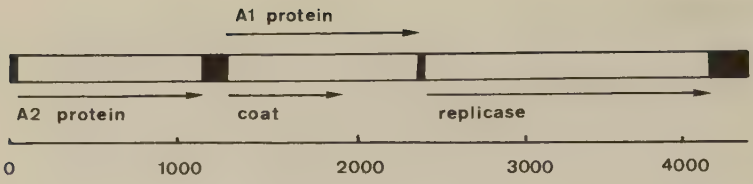


Fig. 1 Map of the Qβ genome: non-translated regions are dark, the locations of the cistrons are marked by arrows.

Two remarkable features should be noted:

1) The genome can be subdivided into translatable and non-translatable sections, as it is found for bacterial and eucaryotic genomes. At both ends of the RNA there exist stretches of at least 60 nucleotides, and between any two cistrons segments of 20 or more nucleotides, none of which are translated into protein (Goodman et al., 1970; Billeter et al., 1969a; 1969b; Hindley et al., 1970; Staples et al., 1971; Staples & Hindley, 1971). The genome of group 1 phages has a similar structure (Nichols, 1970; Jeppesen et al., 1970). The regions immediately preceding the cistrons are thought to be required for the initiation and regulation of protein synthesis. The function of the longer untranslatable segments at the ends of the genome is still unclear. However, mutations generated at the 3'-terminal extracistronic region, by in vitro site directed mutagenesis, suggest that this region is essential for the biological competence of the virus. Alteration in one nucleotide can be lethal (Flavell et al., 1974; Sabo et al., 1977) or cause a selective disadvantage to the mutant phage (Domingo et al., 1976).

2) The utilisation of one cistron for the synthesis of two distinct proteins, coat and A1 protein.

Infectious Cycle

Adsorption: RNA phages infect only so-called male (F^+ or HFr) strains of E.coli (Loeb & Zinder, 1961). These bacteria carry a characteristic appendage, the F^+ pilus, to which the phages can attach in large numbers (Crawford & Gesteland, 1964).

Penetration: After the attachment step the RNA is released from the coat protein shell (Edgell & Ginoza, 1965) by an as yet unknown mechanism and penetrates into the bacterium carrying the maturation protein with it (Krahn et al., 1972, Kozak & Nathans, 1971). None of the phage particle proteins are required for the replication of the virus, since infection by protein-free RNA gives rise to complete, normal phage particles (Strauss, 1964). Synthesis of phage components involves the expression of the phage genome on one hand and the synthesis of progeny phage RNA on the other (see below).

Maturation: Assembly of the phage components occurs very probably spontaneously, without a requirement for additional enzymes and factors. Infectious phage particles could be reconstituted in vitro from isolated phage components (Hofstetter et al., 1974, Roberts & Steitz, 1967).

Lysis: Forty to sixty minutes after infection the host cell is filled with virus particles, partially reassembled phage components and virus-specific side products. Lysis occurs, with the release of 10000 - 40000 particles of which 20 - 50% are infectious. The mechanism of cell lysis is still not understood, however it appears to depend on some unknown function of the phage coat protein (Zinder & Lyons, 1968).

1.2. Synthesis of virus-specific macromolecules

1.2.1 Protein synthesis

After invading its host, the phage RNA serves first as messenger for phage-specific protein synthesis (Godson & Sinsheimer, 1967). The different viral proteins are synthesized in very different molar amounts, commensurate with the phage's requirements for these proteins. Thus in the case of R17, the ratio of synthesis of coat protein to A protein to replicase subunit is about 20 : 2 : 1. How does this regulation come about?

One can distinguish three main regulatory processes:

1) A differential initiation of protein synthesis. The secondary and tertary structure of the viral RNA restricts initiation of protein synthesis at the beginning of the replicase and maturation proteins (A, or A2 for Qβ). Binding and chain initiation occur predominantly at the coat cistron, while initiation at the replicase cistron takes place only while the coat cistron is being translated (Gussin, 1966; Lodish, 1968; Lodish & Zinder, 1966). This is thought to be due to an unfolding of the RNA by the translating ribosomes, which thereby renders the masked binding site at the replicase cistron available for initiation. Initiation at the maturation protein cistron (A, or A2 for Qβ) occurs only as long as the viral RNA is at an early stage of synthesis and becomes unavailable when the nascent strand is further elongated and folded into its mature secondary and tertiary structure (Staples et al., 1971; Robertson & Lodish, 1970; Kolakofsky & Weissmann, 1971 a,b).

2) Suppression of termination: As mentioned before, the middle cistron of Qβ RNA codes for both the coat protein and A1 protein. A1 protein arises by natural translation read-through at the leaky UGA termination signal of the coat protein cistron (Weiner & Weber, 1971, 1973; Horiuchi et al., 1971; Moore et al., 1971).

3) Translational repression: The synthesis of replicase is almost completely turned off about midway through the cycle (Viñuela et al., 1967). This shut down is due to the accumulation of coat protein, which binds at the intercistronic region, preceding the replicase cistron and prevents further initiation of protein synthesis at this point (Eggen & Nathans,1969; Robertson et al., 1968; Ward et al., 1968; Weber, 1976).
A further example of translational repression plays an important role in the early stages of infection. Shortly after infection, the viral RNA is present as a polysome and only after synthesis of the viral polymerase does it become a template for its own replication. The directions of synthesis during translation and replication are opposite to each other on the RNA template and since the replicating enzyme cannot dislodge ribosomes bound to Qβ RNA (Kolakofsky & Weissmann, 1971a), a special mechanism must exist to free the parental RNA of ribosomes and render it competent as template for replication. It was found that Qβ replicase has a strong affinity for the intercistronic region preceding the coat cistron and on binding at this position, prevents the attachment of ribosomes (Kolakofsky & Weissmann, 1971b; Weber et al., 1972). Since ribosome attachment at the A cistron of the mature RNA does not occur to a significant extent (Lodish & Robertson, 1969; Robertson & Lodish, 1970; Staples et al., 1971; Lodish, 1971) and since initiation at the replicase cistron is dependent on translation of the coat cistron (Lodish, 1968; Gussin,1966), the viral RNA is then cleared of ribosomes and becomes available for its own replication.

1.2.2. RNA Synthesis

The replication of viral RNA involves a two step mechanism. The first step is the synthesis of a minus strand, i.e., a strand complementary to the viral RNA (Weissmann & Ochoa, 1967; Weissmann et al., 1968a). The resulting minus strand is then used in a second step as template for the synthesis of progeny phage RNA. Synthesis of both the plus and the minus strand is carried out by one and the same enzyme, replicase (August et al., 1968; Weissmann et al., 1968b).

1) Synthesis of minus strand:

Synthesis starts at the 3'-end of the plus strand (August et al., 1968; Spiegelman et al., 1968). However RNA chain initiation does not occur precisely at the 3'-OH end of the template. Replicase skips the 3'-terminal A of its template to begin at the next nucleotide, a C. The first nucleotide incorporated is thus pppG (Goodman et al., 1970). On the other hand, chain termination does not occur at the template's 5'-end, but an A residue is added to the 3'-end of the product chain (Weber & Weissmann, 1970). This RNA is synthesized as a single stranded structure (Weissmann et al., 1968b), suggesting that the Watson-Crick hydrogen-bonding between the bases of the template and the incoming nucleotides must be undone shortly after the base is incorporated. In addition a host-specific factor, HF1, is required to initiate synthesis of the minus strand (August et al., 1968, Franze de Fernandez et al., 1972).

2) Synthesis of plus strand:

For its synthesis, the host-specific factor, HF1, is not required. Like for the synthesis of minus strand, the 3'-terminal A of the template is not copied (Billeter et al., 1969a,b) and a A residue is added to the completed plus strand (Rensing & August, 1969; Weber & Weissmann, 1970). The RNA is also synthesized in a single stranded form.

The replicating complex, consisting of enzyme, single stranded template and single stranded product is a labile structure, inasmuch as it is easily converted into double stranded RNA, which is completely inactive as template for RNA synthesis (Weissmann et al., 1968b; Feix et al., 1967).

The viral replicase, which has a molecular weight of about 215'000, consists of four different subunits, only one of which, the β subunit, is coded for by the viral genome (Kamen, 1970; Kondo et al., 1970; Fedoroff & Zinder, 1971). The other three are host-specific polypeptides.

The phage-coded subunit (molecular weight 65·000) is responsible for the high specificity of the enzyme to its natural template (Haruna & Spiegelman, 1965) and is most likely responsible for RNA polymerising functions of the enzyme (Landers et al., 1974).

The smaller two subunits are protein synthesis elongation factors EF-Tu and EF-Ts (molecular weight 45·000 and 35·000, respectively) (Blumenthal et al., 1972). They have been implicated in the initiation step of RNA synthesis (Landers et al., 1974; Hori et al., 1974). However, they also appear to be necessary for the RNA chain elongation (Carmichael et al., 1976). Their precise function remains unclear.

The largest subunit (molecular weight 70·000) is 30s ribosomal protein S-1 (Wahba et al., 1974; Kamen et al., 1972) also known as interference factor i (Groner et al., 1972). As part of the replicase molecule, protein S-1 under the normal assay conditions is required for RNA synthesis directed by the viral plus strand RNA, but not by any other templates. Although S-1 lacking replicase has negligible activity with plus strand as template under standard assay conditions, at low ionic strength, S-1 lacking replicase synthesizes high molecular weight RNA at about 1/10 the rate holoenzyme does (Kamen et al., 1972).

The host factor, HF1, has been shown to be a ribosome-associated protein with a high affinity for poly(rA) (Carmichael et al., 1975); its physiological function is unknown. In Qβ RNA replication, the host factor is required for the initiation of plus strand directed RNA synthesis (Franze de Fernandez et al., 1972).

Purified S-1 recognizes two sites in Qβ RNA, one is located near the 3'-end, the second at the intercistronic region preceding the coat cistron. The host factor, HF1, also interacts with Qβ RNA at two sites, one near the 3'-end, the second near the end of the replicase cistron (Senear & Steitz, 1976; Goelz & Steitz, 1977). These results suggest that HF1 and possibly S-1, through their interaction with the 3'-terminal region of Qβ RNA might be

involved in the recognition of the 3'-end of Qβ RNA by replicase. The interaction of S-1 at the intercistronic region preceding the coat cistron might be essential for the repressor activity of Qβ replicase (Weber et al., 1972).

1.3. Template specificity

An important property of Qβ replicase is its template specificity under "physiological" in vitro conditions. Only Qβ RNA (Haruna & Spiegelman, 1965a), its complementary strand (Feix et al., 1968), RNA molecules described as "variants" of Qβ RNA, which had been obtained after propagating Qβ RNA in vitro by replicase for many rounds of synthesis (74 serial transfers) (Mills et al., 1967; Mills et al., 1968), "6s" RNA (Banerjee et al., 1969; Kacian et al., 1971; Prives & Silverman, 1972; Mills et al., 1973; 1975; Schaffner, 1977) or viral RNA of very closely related RNA phages (Miyake et al., 1971) are known to undergo replication in the reaction catalysed by Qβ replicase but not other RNAs, such as R17, f2 or MS2 RNA, E.coli rRNA or tRNA (Haruna & Spiegelman, 1965a; 1965b; Okada et al., 1971).

Poly(rC) and C-rich ribopolymers serve as template for the synthesis of a complementary strand (Hori et al., 1967; Eikhom & Spiegelman, 1967; Feix & Hake, 1975; Küppers & Sumper, 1975), in the presence of Mn^{++} ions, the enzyme will accept many other RNAs as template (Haruna, 1965a; Palmenberg & Kalsberg, 1974; Obinata et al., 1975), but replication of these templates was not achieved.

High concentrations of oligonucleotides complementary to sequences found in the template allow the enzyme to extend complementary synthesis from the 3'-end of the oligonucleotide primer (Feix & Sano, 1975;Vournakis et al., 1977).

What is the molecular basis for the template specificity of Qβ replicase? Although there is no clear - cut answer to this intriguing problem yet, the following considerations have to be

mentioned: Binding studies of replicase to various viral and bacterial RNA molecules (Qβ-, f2-, R23 RNA, ribosomal RNA, tRNA) in the absence of host factor and ribonucleoside - triphosphates demonstrated, whereas replicase binds to Qβ RNA with an association constant of $K_{assoc.} \approx 9.7 \cdot 10^7\ M^{-1}$, it binds other RNA species with a 4 to 17 fold lower affinity (August et al., 1968; Silverman, 1973a; 1973b). This ratio of binding constants is insufficient to account for the enzyme's inability to replicate heterologous templates (Haruna & Spiegelman, 1965b). These findings suggest that other features, beyond binding affinity, are important for a molecule to be active as a template.

A common feature of all templates is the 3'-terminal...$CCC(A)_{OH}$. As initiation of RNA synthesis occurs exclusively at the 3'-terminus of the template (Billeter et al., 1969a; 1969b) and is abolished if the terminal $-C(A)_{OH}$ dinucleotide is removed chemically (Rensing &August, 1969; Weber & Weissmann, 1970) one would expect that the enzyme recognizes the template by its 3'-terminal sequence ...$CCC(A)_{OH}$. However other RNA molecules like MS2 or R17, have identical 3'-termini and yet are inactive as a template (Haruna & Spiegelman, 1965a; Okada et al., 1971). This suggests that the 3C residues at the 3'-terminus may be a necessary but not sufficient feature of active replicase templates.

By comparing nucleotide sequences and secondary structures near the 3'-ends of replicase templates, Küppers proposed that a second cluster of C residues in a defined sterical position close to the 3'-end constituted a key feature for replicase - template recognition (Küppers & Sumper, 1975). As these requirements are not satisfied by Qβ RNA plus strand and certain "6s" RNA variants (Schaffner, 1977), they cannot be a general feature for template recognition by Qβ replicase.

It was found that 3'-terminal fragments of Qβ RNA, function as replicase templates, provided they are longer than about 50% of the total RNA length (Schwyzer et al., 1972). These findings

suggested that in addition to the obligatory 3'-terminal interaction, an internal binding site(s) of replicase on Qβ RNA, located at about the middle of the genome is required for the template recognition process.

The internal binding site at the intercistronic region preceding the coat cistron (Fig. 1), which is located some 900 nucleotides upstream of the middle of the genome, is therefore not essential for replication and seems to be only involved in repression of protein synthesis (Weber et al., 1972).

Considering that replicase has a low affinity for the 3'-terminus itself (Weber et al., 1972), it was postulated that template recognition by replicase involves the binding at an internal site on the RNA (located between the 3'-end and the middle of the genome) and the enzyme positions the 3'-end at the "initiation site" where chain initiation occurs (Schwyzer, 1973). This model accounts for the fact that other RNAs, such as MS2 or f2 RNA, which have relatively high affinities for Qβ replicase and have C-rich 3'-termini similar to that of Qβ RNA, do not allow any initiation of RNA synthesis. Within the framework of the model, replicase is thought to bind to an internal region of the RNA for which it has some affinity, however due to the tertiary structure of the RNA, its 3'-end cannot be positioned into the "initiation site".

The present work offers evidence that, at least in vitro, replicase interacts at a second internal site on Qβ RNA, which is located around the middle of the genome. The portions of Qβ RNA that bind to the replicase have been identified by isolating the RNA segments of the binding complex. The complete nucleotide sequence of the major RNA segments is presented as well as the results of several experiments, illustrating the functional role of internal binding sites in template recognition.

2. MATERIALS AND METHODS

2.1. Materials

Qβ replicase, purified according to Kamen et al. (1972), was a gift from Prof. Dr. C. Weissmann. The specific activity, measured on Qβ RNA was 1900 units/mg (WR 323), used for the binding studies in sections 3.1., 3.2., 3.4., 3.6., 3.7., 3.8.; 1400 units/mg (TW 335A) used in section 3.5. and 1200 units/mg used in section 3.3. Host factor was a gift from Prof. Dr. C. Weissmann. The preparation TW 335A was used in section 3.5. and preparation TW 327B was used in sections 3.7. and 3.8. ^{32}P-and ^{3}H-labeled Qβ RNA were prepared according to Weissmann et al. (1968) with some modifications (Escarmis, C., Billeter, M.A. & Weissmann, C., unpublished).

Replicative intermediate and Qβ RNA were a gift from Prof. Dr. C. Weissmann. Pancreatic RNase A was purchased from Worthington, RNase U_2 was from Sankyo. RNase T_1 (Calbiochem.), treated with acid EDTA according to Dahlberg (1968), was a gift from Dr. M.A. Billeter; partially purified RNase T_2, prepared from Taka Diastase (Sankyo) as described by Hiramaru et al. (1966), was a gift from Dr. H. Weber. Pronase B grade (Calbiochem.), incubated at 20 mg/ml in 5 mM EDTA, 10 mM Tris-HCl (pH 7.5) for 90 min at 37°C to destroy RNase activity, was a gift from Dr. M.A. Billeter. An extracellular ribonuclease from Physarum Polycephalum was a gift from Dr. J.P. Bargetzi (University of Geneva). Carboxymethylated pancreatic ribonuclease was a gift from Dr. W. Fiers (University of Ghent). Yeast carrier RNA (British Drug Houses, Pool, England) was rendered free of RNase by extraction with phenol and precipitation with ethanol. BSA, fraction V, B grade (Calbochem.) was shaken with macaloid (Baroid National Lead Co., Hamston, Texas) to remove RNase activity and dialysed for several days against distilled water.

α-^{32}P-labeled nucleoside triphosphates were a gift from Dr. M.A. Billeter. Unlabeled ribonucleoside triphosphates (sodium salt) were from Boehringer or Schuchardt. Phenol was distilled and stored at -20°C. SDS was recrystallised from aqueous ethanol prior to use. Acrylamide (puriss) and N'N methylene bisacrylamide were from Serva, Germany. DEAE cellulose paper was from Whatman and cellulose acetate strips (3 x 57 cm) were from Schleicher and Schuell. Nitrocellulose filters of 0.45 μ pore size and 25 mm diameter (type HAW P 25) were from Millipore Co.

Rabbit globin mRNA was a gift from Dr. T. Staehelin (Basel Inst. for Immunology).

Poly(rC) and Poly(rU) were purchased from Calbiochem.

E.coli ribosomal RNA was a gift from Dr. H. Weber.

2.2. Methods

2.2.1. General procedure for the isolation of Qβ RNA fragments, bound by Qβ replicase

In principle, Qβ replicase was bound to ^{32}P-labeled Qβ RNA under different ionic conditions. The binding mixture was digested by RNase T_1 in the presence of unlabeled Qβ RNA (to reduce unspecific binding of ^{32}P-labeled RNA fragments to Qβ replicase) and the RNA fragments retained by the enzyme were isolated on a Millipore filter, as described by Weber et al. (1972) with some modifications.

The binding of ^{32}P-labeled Qβ RNA to replicase was performed at a mass ratio of Qβ RNA to replicase of about 2 (the molar ratio of replicase to RNA was therefore 6.2 : 1), at RNA concentrations of 0.1 - 0.2 mg/ml and 0.05 - 0.1 mg/ml respectively, using four sets of ionic conditions:

a) 10 mM Tris - HCl (pH 7.5), 10 mM $MgCl_2$, 0.2 mg/ml BSA, 2 mM DTT (= M-conditions).

b) 80 mM Tris - HCl (pH 7.5), 12 mM $MgCl_2$, 1 mM EDTA, 0.2 mg/ml BSA, 2 mM DTT (= I-conditions).

c) 10 mM Tris - HCl (pH 7.5), 150 mM NaCl, 0.2 mg/ml BSA, 2 mM DTT (= S-conditions).

d) 80 mM Tris - HCl (pH 7.5), 8 mM $MnCl_2$, 0.2 mg/ml BSA, 2 mM DTT (= Mn-conditions).

After 4 - 8 min incubation at 37°C, the binding mixture were diluted into 5 volumes of the same buffer (lacking BSA and DTT) containing a 5 fold excess of unlabeled Qβ RNA over radio - actively labeled Qβ RNA, which had been adjusted to 25°C, by preincubation for 15 min at 25°C. RNase T_1 (1 unit RNAse T_1 / µg RNA) for the samples a, b and d; and 0.5 unit RNase T_1 / µg RNA for sample c) was added and incubation was continued for 2 min (sample c) or 10 min (samples a, b and d) respectively. These digestion conditions were chosen for reasons which are outlined in section 3.1.

The samples were then filtered under mild suction onto a Milli - pore filter disc (Ø 2.4 cm; pore size 0.45 µ), which had been presoaked in the corresponding binding buffer (without BSA and DTT). The filter was rinsed three times with 1 ml of buffer, such that the whole surface of the filter was completely covered with liquid. The ^{32}P radioactivity retained on the filter, as determined by measuring the Čerenkov radiation, comprised about 3 - 3.5% of the ^{32}P-RNA initially added in the case of M- and I-conditions, about 1% under S-conditions and about 4 - 5% under Mn-conditions.

To prepare 0.1 nmole quantities of enzyme-protected RNA fragments, this procedure was carried out several times. Batches of up to 5 filters were extracted by shaking for 20 min at room temperature in 5 ml of a 1 : 1 mixture of extraction buffer (20 mM Tris - HCl

(pH 7.5), 2 mM EDTA, 0.5% SDS) and phenol (saturated with extraction buffer before use). The samples were heated to 50°C for about 3 min with intermittent shaking, then cooled to 0°C and centrifuged at 10'000 rpm. The aqueous phase, containing 80 - 90% of the filter-bound RNA was removed and adjusted to 1 x TNE. 20 µg yeast carrier RNA were added and the RNA was precipitated by adding two volumes of ethanol and storing at -20°C for at least 5 hours. In some experiments the extraction was performed in the presence of 20 µg yeast carrier RNA, without finding any difference in the recovery of the filter-bound RNA. The precipitate was collected by centrifugation, washed once with 70% ethanol and dried in vacuo.

A) Separation of replicase-bound RNA fragments by one dimensional polyacrylamide gel electrophoresis.

The dried RNA precipitates were dissolved in 30 µl of 12% sucrose containing 0.05% bromophenol blue, and the samples were layered in 15 mm wide slots of a 20% polyacrylamide slab gel (dimensions: 40 x 20 cm;thickness: 4 mm for preparative, 2 mm for analytical gels). The electrophoresis conditions were based on those described by Peacock and Dingman (1967).The gel was polymerised from a solution with the following composition: 20% acrylamide, 0.8% bisacrylamide; 90 mM Tris-base, 90 mM boric acid, 2.5 mM EDTA; 0.36% dimethylaminopropionitrile, 0.05% ammonium persulfate. The gel was placed vertically in a glass tank containing electrophoresis buffer (90 mM Tris-base, 90 mM boric acid, 2.5 mM EDTA (pH 8.3)) up to the level of the gel surface. A wick consisting of 6 layers (preparative gels) or 3 layers (analytical gels) of Whatman 3MM wrapped in Saran foil was used. Electrophoresis was carried out at 800 V (60-70 mA) at 4°C, until the dye had moved at least 25 cm. The moist gel was autoradiographed, the gel regions corresponding to the radioactive bands were cut from the gel, crushed by extrusion through disposible 3 ml plastic syringes and extracted with 3-4 volumes of 0.4 mM NaCl for

20 min at 37°C. The suspension was centrifuged at 1500 rpm for 5 min, the clear supernatant was taken off and the polyacrylamide sediment was reextracted twice as above. The combined supernatants, containing 70-90% of the ^{32}P-radioactivity were filtered through Sephadex G-10 in a pasteur pipette (bed volume 50-100 µl) equilibrated with 0.4 M NaCl. The RNA was precipitated with two volumes of ethanol and stored at -20°C.

B) RNase T_1 digestion of replicase bound RNA fragments and fractionation of the digestion products by two-dimensional polyacrylamide gel electrophoresis.

The dried RNA precipitates were dissolved in 8 µl 20 mM Tris-HCl (pH 7.5), 2 mM EDTA and digestion with RNase T_1 was performed with 0.5 units RNase T_1 per µg RNA for 30 min at 37°C. The digestion products were fractionated by two dimensional polyacrylamide gel electrophoresis as described by De Wachter and Fiers (1972). After autoradiography the gel pieces corresponding to the radioactive spots were cut from the gel, their radioactivity determined by measuring the Čerenkov radiation and the RNA extracted for analysis according to Billeter, unpublished. The quantitation of the various oligonucleotides was performed by calculating the cpm per nucleotide for each oligonucleotide.

2.2.2. Preparation of random RNA fragments and homopolymer chains of discrete size

1. Digestion of the various RNA species.

In order to prepare RNA fragments and homopolymer chains having a size range between 100 and 200 nucleotides, the following digestion conditions were used:
a) Unlabeled and ^{32}P-labeled Qβ RNA: The RNA was incubated at a concentration of 0.5 mg/ml in 50 mM Na_2CO_3 for 50-80 min at 50°C.

b) E. coli ribosomal RNA was incubated at a concentration of 0.5 mg/ml in 50 mM Na_2CO_3 for 20-30 min at 50°C.
c) Poly(rC) was digested for 1 min at 50°C at a concentration of 2 mg/ml in 100 mM NH_4HCO_3 (pH 10).
d) Poly(rU) was incubated for 5 min at 50°C at a concentration of 1.4 mg/ml in 0.3 M NH_4HCO_3 (pH 10).
The reagents used in the reactions c and d were preincubated for 5 min at 50°C.
The samples were immediately chilled in ice, and 0.8 M NaCl, 0.5 M Tris-HCl (pH 7.5) was added (1/4 volume). The RNA was precipitated with 2 volumes of ethanol at -20°C overnight.

2. Fractionation of the various RNA fragments.

The precipitates were collected by centrifugation, washed once with 70% ethanol, redissolved in 100 µl 12% sucrose containing 0.05% bromophenol blue and split into two halves. 200 µg of RNA in 50 µl were applied per 4 x 15 mm slot of a 20% polyacrylamide slab gel. Electrophoresis was carried out as described above. Radioactively labeled RNA fragments of known sizes (^{32}P-labeled M-2a, 165 nucleotides long and ^{32}P-labeled S-3,100 nucleotides long) were coelectrophoresed in neighboring slots as markers.
From the unresolved RNA fragment patterns, gel pieces corresponding to the length of fragments M-2a and S-3 were cut from the gel and the RNA extracted as above. In the case of the homopolymers, a broader size range corresponding to a chain length of 130 ± 20 nucleotides was isolated.
For the experiments described in section 3.4, unlabeled fragmented Qβ RNA, prepared as described above, was fractionated by 2 subsequent sucrose gradient centrifugations. The fractions having an average length of M-2a (165 nucleotides) and S-3 (100 nucleotides), respectively were selected.

3. Size determination of poly(rC).

Poly(rC) used as a template for the poly(rC) directed synthesis (section 3) was prepared as follows:
Poly(rC) ($s_{20,w}$ = 8-13s), was fractionated on a preparative scale by centrifugation through a 5-23% sucrose density gradient in 50 mM Tris-HCl (pH 7.5), 5 mM EDTA for 6 h at 55'000 revs/min and 15°C, in the Spinco SW 60 rotor. The peak fractions were pooled and the chain lengths of poly(rC) were determined by polyacrylamide gel electrophoresis under denaturing conditions. E.coli 5 s RNA, yeast t RNA, 10 s rabbit globin mRNA, ^{32}P-labeled Qβ RNA fragments having 165 and 100 nucleotides in length, respectively, were used as size markers. Electrophoresis was carried out in a polyacrylamide slab gel (20 x 20 x 0.2 cm) containing 15% acrylamide, 0.5% bisacrylamide; 90 mM Tris base, 90 mM boric acid, 2.5 mM EDTA; 0.06% ammonium persulfate and 0.03% TEMED in 7 M urea prepared and run essentially according to Maniatis et al. (1975).The samples were heat denatured and applied on the gel as described by Escarmis, C., Billeter, M. A. and Weissmann, C. (unpublished).Electrophoresis was at 25 mA (400 V) for 8 h at room temperature. After staining for 10 min with 0.02% aqueous methylene blue, the gel was autoradiographed. The position and the intensity of the stained RNA bands were determined by densitometer tracing. The electrophoretic mobilities of the various size markers were plotted versus the log of their molecular weights (linear function) and the size distribution of the poly(rC) could be evaluated by interpolation. The chain lengths ranged from 200-550 nucleotides with a number average molecular weight of 1 x 10^5.

2.2.3. Total digestion of ^{32}P-labeled Qβ RNA with RNase T_1 on a preparative scale

^{32}P-labeled Qβ RNA (260 µg) was incubated in 25 µl of 20 mM Tris-HCl (pH 7.5), 2 mM EDTA containing 160 units of RNase T_1

for 30 min at 37°C. 0.3 ml of TNE and 0.3 ml of TNE-saturated phenol was added and the preparation was twice extracted with phenol. The RNA was precipitated with 2 volumes of ethanol at -20°C. The precipitate was collected by centrifugation (40 min at 10'000 rpm) and washed once with 70% ethanol. The dried residue was dissolved in water to give a RNA concentration of 1 mg/ml. 70-75% of the input radioactivity were recovered.

2.2.4. Preparation of radioactively labeled Qβ RNA by in vitro synthesis

^{32}P-labeled Qβ RNA was synthesized in presence of one α-^{32}P-labeled and three unlabeled nucleoside triphosphates. A preparation for which α-^{32}P GTP was used is described in detail: The incubation mixture contained in 1 ml: Qβ replicase (30 units), Qβ RNA (32 µg), host factor I (30 units), 0.14 mM α-^{32}P GTP (10^7 cts/min per nmole), 0.8 mM each of CTP, UTP and ATP, 0.16 M KCl, 80 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 1 mM EDTA. Incubation was for 45 min at 37°C. The incorporation into acid-insoluble material was 28 nmoles of ^{32}P GMP (40 µg of ^{32}P RNA).
The reaction mixture was treated for 15 min at 37°C with 400 µg pronase in presence of 0.2% SDS and twice extracted with phenol. The RNA was precipitated with ethanol and dissolved in 0.25 ml TNE. The solution was chromatographed at 4°C through a column (ø 0.8 cm) consisting of Chelex-100 (Na^+) (bed volume 0.3 ml), overlayered with Sephadex G-50 (bed volume 9 ml), equilibrated with TNE. The fractions corresponding to the excluded peak were identified by Čerenkov radiation, pooled and the RNA precipitated with 2 volumes of ethanol. (recovery: 23 nmoles of ^{32}P GMP, 32 µg ^{32}P RNA). An aliquot of the product in 100 µl 1 mM EDTA was mixed with authentic ^{3}H Qβ RNA, denatured for 90 sec at 100°C and analysed by centrifugation through a 5-23% sucrose density gradient in 50 mM Tris-HCl (pH 7.5) and 5 mM EDTA for 2 h at 60'000 rpm and 15°C, in a Spinco SW 65 rotor.

18-20 fractions were collected on glass filters and the ^{3}H and ^{32}P-radioactivities were determined. Preparations containing a significant amount of 6 s RNA as a contaminant of the 30 s product were centrifuged through a sucrose density gradient on a preparative scale and the peak fractions sedimenting at 30 s were pooled, adjusted to 1 x TNE and the RNA precipitated with 2 volumes of ethanol. The final preparations consisted generally of about 60-65% plus strand and 35-40% minus strand RNA, as determined by an annealing assay (W. Müller, personal communication). Since the presence of minus strands was found not to interfere with the isolation of the replicase bound plus strand RNA fragments under the conditions used, no attempts were made to purify plus strand RNA.

2.2.5. Sequence determination

a) Fingerprinting of the ^{32}P-labeled RNA fragments M-2a, M-2b and characterisation of the resulting oligonucleotides.

The purified ^{32}P-labeled RNA fragments M-2a, M-2b from the M-site were digested with RNase T_1 and RNase A, respectively and the resulting oligonucleotides were separated by two-dimensional electrophoresis on DEAE-paper according to Sanger et al. (1965). In cases, when many samples had to be analysed at the same time, this procedure was performed on a smaller scale, as described by Schaffner (1977). 8 samples could be processed simultaneously. The radioactivity in the oligonucleotide spots was determined, the oligonucleotides were extracted and their nucleotide sequence was determined essentially according to Brownlee (1972).

Oligonucleotides obtained by RNase T_1 digestion were subjected to cleavage by:

1) RNase A: 1 µg RNase A per µg RNA in 10 µl 20 mM Tris-HCl (pH 7.5), 2 mM EDTA; incubation, 30 min at 37°C.

2) RNase U_2: 1 unit/ml per 10 µg RNA in 10 µl 50 mM NaAc (pH 4.5),

2 mM EDTA, 0.1 mg/ml BSA; incubation, 2 h at 37°C.
3) Mononucleotide analysis was performed by digestion with 50 units/ml of RNase T_2 and 400 µg/ml of RNase A in 10 mM NaAc, 1 mM EDTA (pH 4.5) for 1 h at 37°C.
Oligonucleotides obtained by RNase A digestion were analysed by:
1) RNase T_1 digestion: 1 unit RNase T_1 per µg RNA in 10 µl 20 mM Tris-HCl (pH 7.5), 2 mM EDTA; incubation, 30 min at 37°C.
2) Mononucleotide analysis, as described above.
The cleavage products were identified by their mobilities relative to those of standard digestion products and, where necessary by base analysis.

Some T_1 oligonucleotides were digested partially by RNase U_2: The sample was incubated with 0.8×10^{-3} units of RNase U_2 for 30 min at 37°C in 4 µl buffer containing 25 mM NaAc (pH 4.5), 1 mM EDTA, 50 µg/ml BSA and 5 mg/ml of carrier RNA. The products were separated in two dimensions on cellulose acetate and DEAE-paper. The partial digestion products were further analysed by total digestion with RNase U_2 and/or RNase A.

In some cases ribonuclease I from Physarum polycephalum, which cleaves UpN and ApN more rapidly than CpN linkages (Pilly et al., 1977), was used. Digestion was carried out in 10 µl of 0.1 M NaAc (pH 5.0), 1 mM EDTA, 50 µg/ml BSA, 3.75 units/ml of ribonuclease and 1 mg/ml of RNA at 37°C from 30-100 min, depending on the nature of the oligonucleotide, and aliquots were removed at 2 different times. The digest was fractionated by electrophoresis on DEAE-paper at pH 1.9. In some cases the products were only characterised by digestion with RNase T_2 and in others the products were first dephosphorylated and then the total nucleotide composition as well as the 3'- and 5'-terminal nucleotides were determined by incubating 2 samples with RNase T_2 and snake venom exonuclease respectively, as described by Escarmis, C., Billeter, M. A. and Weissmann, C., unpublished.

b) Partial digestion of the ^{32}P-labeled RNA fragments M-2a and M-2b.

Overlapping degradation products of various sizes (11-100 nucleotides in length) of the fragments M-2a and M-2b were obtained by partial digestion with RNase T_1 or carboxymethylated RNase A.

1) Partial digestion with RNase T_1: The digestion was performed in 20 µl 20 mM Tris-HCl (pH 7.5), 2 mM EDTA at 0°C. Several enzyme to substrate ratios were used in parallel, in order to obtain a broad size distribution of various digestion products. The following reaction conditions were used:

a) 0.05-, 0.1-, 0.15 units of RNase T_1 per µg RNA containing 2.5 mg/ml of yeast carrier RNA; incubation time, 55 min.

b) 0.3-, 0.5 units of RNase T_1 per µg RNA containing 6.25 mg/ml of yeast carrier RNA; incubation time, 50 min.

c) 0.23 units of RNase T_1 per µg RNA containing 2.5 mg/ml of yeast carrier RNA; incubation time, 50 min.

2) Partial digestion with carboxymethylated RNase A:

Partial digestion was carried out with 0.8 µg carboxymethylated RNase A in 20 µl of buffer containing 50 mM NaAc, 2 mM EDTA, 0.04% gelatine (pH 4.0) and 1.25 mg/ml of carrier RNA, for 33 min at 10°C.

All partial degradation reactions were stopped by adding 0.4 ml of a solution containing 20 mM Tris-HCl (pH 7.5), 2 mM EDTA and 0.5% SDS. The RNA was purified by extracting twice with phenol at room temperature. The aqueous phases were adjusted to 1 x TNE and precipitated with 2 volumes of ethanol. The precipitate was collected by centrifugation, dissolved in 40 µl 1 mM EDTA and heat denatured for 90 sec in a boiling water bath. After rapid cooling, the solution was adjusted to 12% sucrose containing bromophenol blue and applied onto a 20% polyacrylamide gel, as described in section 2.2.1.A. Electrophoresis was carried out at 800 V (60-70 mA) for 15 h.

After autoradiography, gel slices corresponding to the radioactive bands were cut from the gel and ground with a tightly fitting, roughened glass pestle, as described by Schaffner (1977). After extraction for 20 min at 37°C with 3-4 volumes of 0.5 M NaCl, the polyacrylamide was pelleted by centrifugation and the extraction was repeated. The combined supernatants, containing 70-90% of the ^{32}P-radioactivity, were filtered through Sephadex G-10 (bed volume 0.1 ml), equilibrated in 0.5 M NaCl (to remove residual fine polyacrylamide particles). Yeast carrier RNA (50 µg) was added and the RNA precipitated with 2 volumes of ethanol. The major carboxymethylated RNase A partial digestion products were further fractionated by electrophoresis on a 15% polyacrylamide gel in 7 M urea prepared and run as described in section 2.2.2.3. The RNA was extracted as described above. The partial digestion products were analysed by RNase T_1 and RNase A fingerprinting on DEAE-paper on a small scale as described by Schaffner (1977).

3. RESULTS

3.1. Association of Qβ replicase and Qβ RNA

Binding of replicase to Qβ RNA was analysed by retention of the RNA-enzyme complex to a Millipore membrane filter (August et al., 1968). Most proteins bind to nitrocellulose filters, whereas free RNA or DNA do not (August et al., 1968; Thang et al., 1968; Yarus & Berg, 1970; Hinkle & Chamberlin, 1972). Complexes between proteins and RNA or DNA can be quantitatively determined by using a radioactively labeled RNA or DNA and measuring the radioactivity bound to the filter.

It was known from earlier studies on the binding of the enzyme to RNA that replicase interacts with Qβ RNA in the absence of components that are required for the initiation of synthesis (August et al., 1968). Previous results had furthermore shown that the interaction of replicase on Qβ RNA was sensitive to the ionic conditions used in the binding mixture (Weber, H., Kamen, R. & Weissmann, C., unpublished experiments). Binding replicase to Qβ RNA in the presence of Mg^{2+} (M-conditions: 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$) or salt (S-conditions: 10 mM Tris-HCl (pH 7.5), 150 mM NaCl) resulted in two different binding patterns.

I first studied the saturation of the enzyme-binding sites by Qβ RNA under these two different ionic conditions.

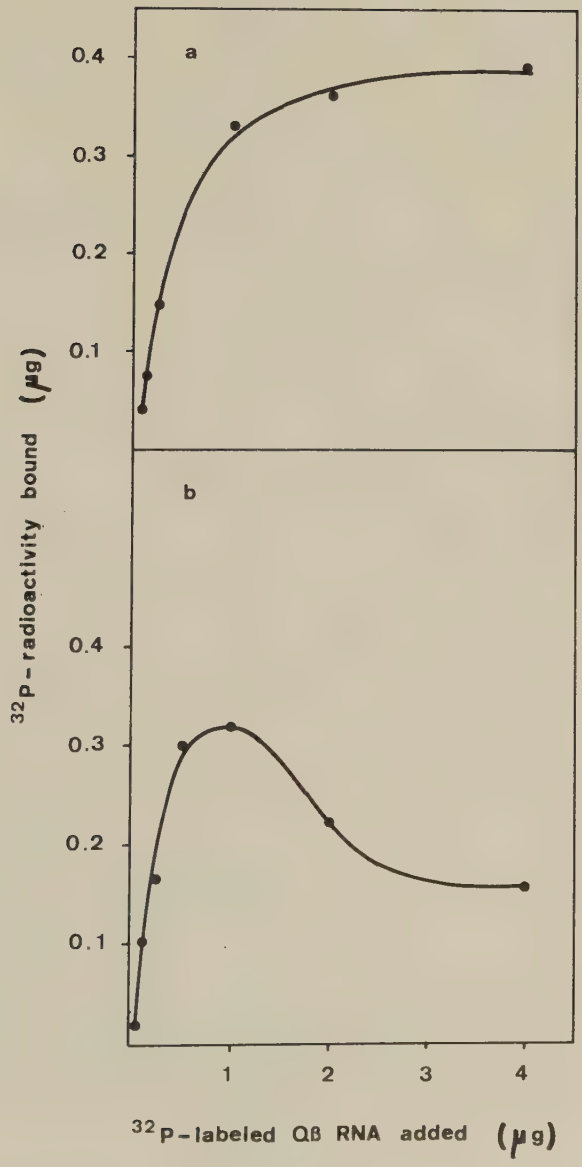
Binding curves of the enzyme to RNA are shown in Fig. 3.1. At saturating amounts of RNA (ratio of RNA: enzyme = 8, by weight) under M-conditions (Fig. 3.1.a), 0.26 pmoles of RNA are bound per pmole of replicase present. This low saturation point might be due to a low efficiency of the binding of the complex to the filter (Hinkle & Chamberlin, 1972).

Under S-conditions (Fig. 3.1.b) the binding curve reaches a maximum at 0.2 pmoles of RNA bound per pmole of enzyme present at an RNA: enzyme ratio of about 4 (by weight). At higher ratios

Figure 3.1 Binding of Qβ RNA to replicase under various ionic conditions.

a) M-conditions: Qβ replicase (0.22 μg) was incubated for 8 min at 37°C with the indicated amounts of ^{32}P Qβ RNA (specific activity: 1.2 10^6 cpm/pmole) in 10 μl of 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.2 mg/ml BSA, 2 mM DTT. The reaction mixtures were then chilled, 0.2 ml of washing buffer (10 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5)) were added and the mixtures were filtered through Millipore filters. The filters were washed 3 times with 1 ml of washing buffer, dried and the radioactivity was measured in a toluene-PPO-POPOP scintillator solution. The radioactivity retained on the filter in the absence of replicase (0.1% of the input radioactivity) was subtracted from each point.

b) S-conditions: the same as a) except that 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 0.2 mg/ml BSA, 2 mM DTT was used as biding buffer and 150 mM NaCl, 10 mM Tris-HCl (pH 7.5) was used as washing buffer. The radioactivity retained on the filter in the absence of replicase (0.1% of the input radioactivity) was subtracted from each point.


a
b
0.4
0.3
0.2
0.1
0.4
0.3
0.2
0.1
1
2
3
4
^{32}P - radioactivity bound (µg)
^{32}P - labeled Qß RNA added (µg)

this value drops again. This binding behaviour could mean that at lower ratios of RNA to enzyme (when the enzyme is in large excess over the RNA), several enzyme molecules are bound per RNA molecule and these complexes might have a higher binding efficiency to the filter than the ones obtained at higher ratios (when the RNA is in excess over the enzyme), where presumably only one enzyme molecule is bound per RNA molecule. Although the replicase-RNA complex has been considered to have a 1:1 stoichiometry (August et al., 1968), it was shown that as many as 30 enzyme molecules can bind to each molecule of Qβ RNA and that most, if not all, of the molecules bind with the same affinity (Silverman, 1973b).

The regions of Qβ RNA which are bound to Qβ replicase under these different ionic conditions were identified using the procedure described by Weber et al., (1972) and Weber, H., Kamen, R. & Weissmann, C., unpublished results.
Qβ replicase was bound to ^{32}P-labeled Qβ RNA and the enzyme-RNA complexes were digested by RNase T_1. A five fold excess of unlabeled Qβ RNA was added prior to nuclease digestion in order to diminish any unspecific binding of digested ^{32}P-labeled RNA to replicase. Oligonucleotides remaining bound to the enzyme were isolated by adsorption of the complexes to Millipore filters, eluted from the filters and fractionated by electrophoresis on a 20% polyacrylamide gel (Fig. 3.2). The binding complexes used for nuclease degradation were prepared using RNA-enzyme ratios approaching saturation according to the binding curves (Fig. 3.1), i.e. about 2:1 by weight. At this ratio, about 50-60% of the input RNA bound to Millipore filters prior to degradation. Digestion conditions were selected such as to reduce the amount of bound RNA to 1% for S-conditions and to 3% for M-conditions.Control experiments showed that increasing the RNA/protein ratio to 16 (by weight) did not result in any changes in the fragment pattern. The digestion conditions were chosen such as to give rise to a reproducible pattern of RNA

bands on a polyacrylamide gel without any undigested RNA remaining at the origin. It should be noted that RNA segments recovered under these conditions are not truly protected but are rather partial degradation products remaining selectively attached to the enzyme. Digestion with higher amounts of nuclease leads to a further decrease in the yields of these bands, although the overall pattern remains essentially the same (Weber, H., unpublished results).

Fig. 3.2.a shows the band pattern obtained under S-conditions. The characterisation of these RNA segments and the possible function of the interaction at their region of origin, the so-called S-site, has been described by Weber et al. (1972).

Under M-conditions, the band pattern looked more complex (Fig. 3.2.b). The complete nucleotide sequence of the most prominent bands designated M-2a, M-2b has been established in the present work. Whereas the complete nucleotide sequences of M-5 and M-11 have also been determined (Weber, H., unpublished results), M-7, M-8, M-9 were not further analysed because they appeared to be mixtures of several RNA species or their appearance was not reproducible.

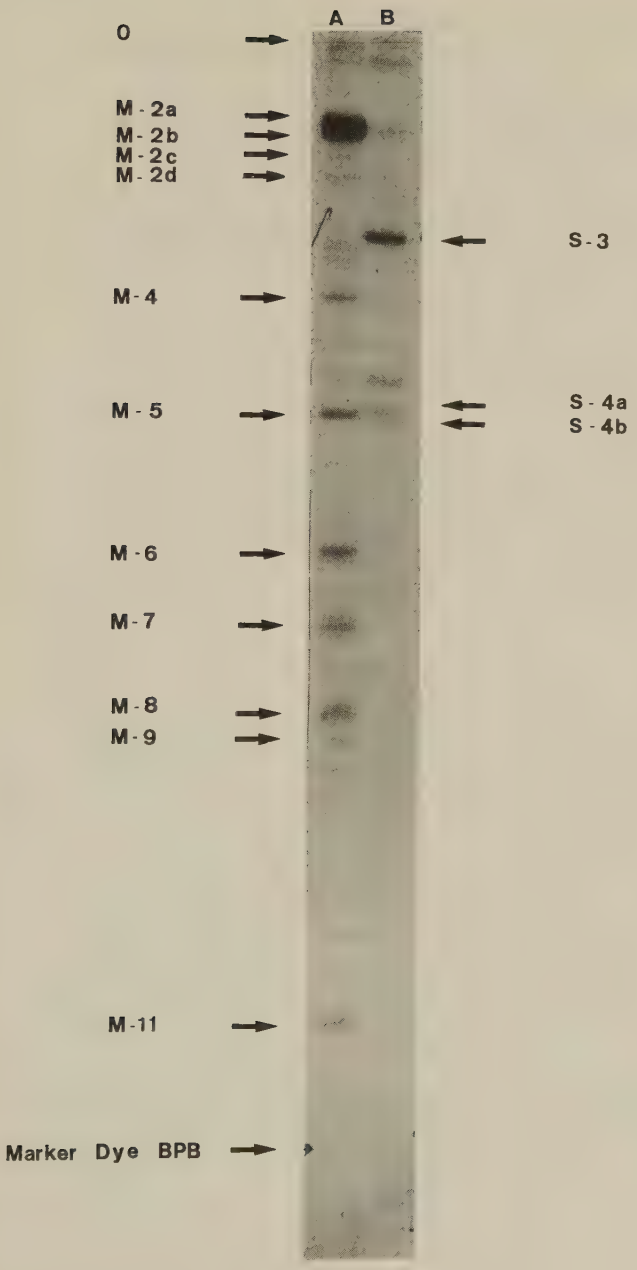
The length of the various fragments ranges from 173 nucleotides (M-2b) to 22 nucleotides (M-11). RNase T_1 fingerprints revealed that all fragments obtained under M-conditions (except M-2a, b, c, d) are non-overlapping, since they did not show any significant oligonucleotide spots in common.

About 15% of the total radioactivity applied to the gel were present in the bands M-2a/b, 3.5% in M-5 and 1.7% in M-11. The recovery of these RNA segments was 0.05 pmoles (M-2a/b), 0.028 pmoles (M-5) and 0.046 pmoles (M-11) per pmole of replicase and 0.3 pmoles of Qβ RNA present in the binding mixture.

Furthermore among the major bands present there was no fragment derived from the S-site.

These data show that there are at least two different

Figure 3.2 Electrophoretic analysis of ^{32}P Qβ RNA fragments remaining bound to Qβ replicase after digestion with RNase T_1.
^{32}P-labeled Qβ RNA (100 μg, specific activity: 3.8 10^3 cpm/μg) and Qβ replicase (45 μg) were incubated for 8 min at 37°C in 0.5 ml binding buffer. The binding complex was digested by RNase T_1, replicase-bound RNA fragments were retained on Millipore filters, recovered, fractionated by 20% polyacrylamide gel electrophoresis, and an autoradiograph was prepared. The detailed procedure is described in the Methods section (2.2.1).
A) Oligonucleotide pattern obtained after binding under M-conditions (10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$): M-site.
B) Oligonucleotide pattern obtained after binding under S-conditions (10 mM Tris-HCl (pH 7.5), 150 mM NaCl): S-site.


A
B
O
M-2a
M-2b
M-2c
M-2d
S-3
M-4
M-5
S-4a
S-4b
M-6
M-7
M-8
M-9
M-11
Marker Dye BPB

interactions of replicase on Qβ RNA:
1) one which is salt dependent and does not require Mg^{2+}-ions (S-site).
2) one which is Mg^{2+} dependent, located at a different region on Qβ RNA (M-site). It will be shown later that M-fragments are derived from a circumscribed region.
Binding of replicase to Qβ RNA in the presence of Mg^{2+} and salt yields a band pattern comprising fragments of both sites (Weber, H., unpublished results).

3.2. Sequence determination of the bound RNA fragments M-2a and M-2b

In order to get some insight regarding which structural features might be involved in the recognition process between replicase and Qβ RNA, the complete nucleotide sequence of the predominant RNA fragments M-2a and M-2b was determined.
In order to establish the RNase T_1 and RNase A oligonucleotide catalogs of these RNA fragments, the nearest neighbour technique was used. For this purpose four samples of Qβ RNA were prepared in vitro, using in turn either α-^{32}P UTP, α-^{32}P GTP, α-^{32}P CTP, α-^{32}P ATP as the only labeled precursor. In addition uniformly ^{32}P -labeled Qβ RNA was obtained from virus, labeled in vivo. RNA segments protected by replicase under M-conditions were prepared (see Methods section) for each of the 5 RNA preparations. The RNA fragments M-2a, M-2b were extracted from the polyacrylamide gels and aliquots of each sample were digested with RNase A and RNase T_1, respectively. The resulting oligonucleotides were separated by two dimensional electrophoresis (Sanger et al., 1965). Autoradiograms of the RNase T_1 and RNase A fingerprints of the RNA fragments M-2a and M-2b are shown in figures A-1 to A-4.
The various digestion products were further analysed to establish their respective sequences as described in the Methods section.

Figures A-1 to A-4 Autoradiograms of two-dimensional fingerprints of the fragments M-2a and M-2b.

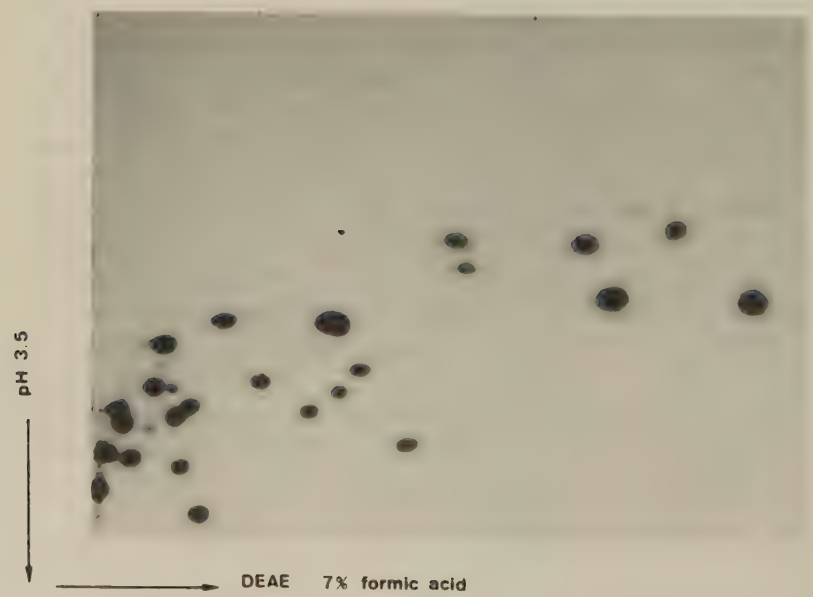
A-1 and A-2: Autoradiograms of RNase T_1 digestion products of fragments M-2a (A-1), and M-2b (A-2).
First dimension: Electrophoresis on cellulose acetate in 7 M urea at pH 3.5.
Second dimension: Electrophoresis on DEAE-paper in 7% formic acid.

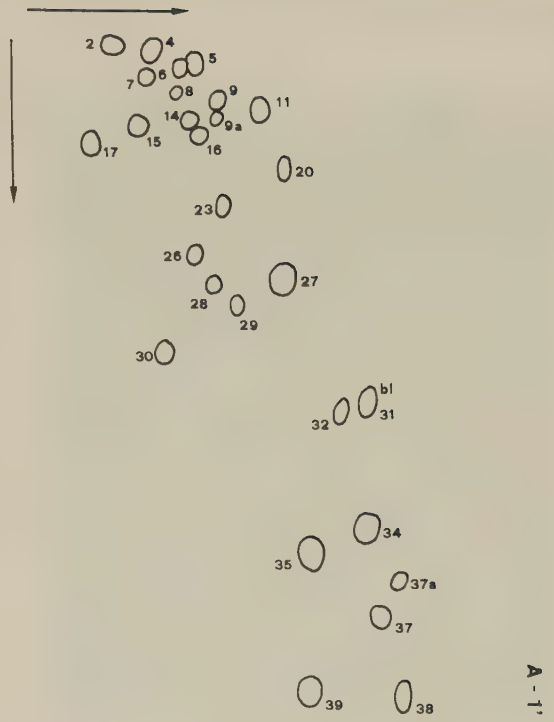
A-3 and A-4: Autoradiograms of RNase A digestion products of fragments M-2a (A-3), and M-2b (A-4).
First dimension: Electrophoresis on cellulose acetate in 7 M urea at pH 3.5.
Second dimension: Electrophoresis on DEAE-paper at pH 1.9.

A-1' to A-4': Tracings of A-1 to A-4.
The numbered spots are identified in Tables 3.1 and 3.2.
The symbol bl indicates the position of the blue dye marker (xylene cyanol FF).

A-2

A-1

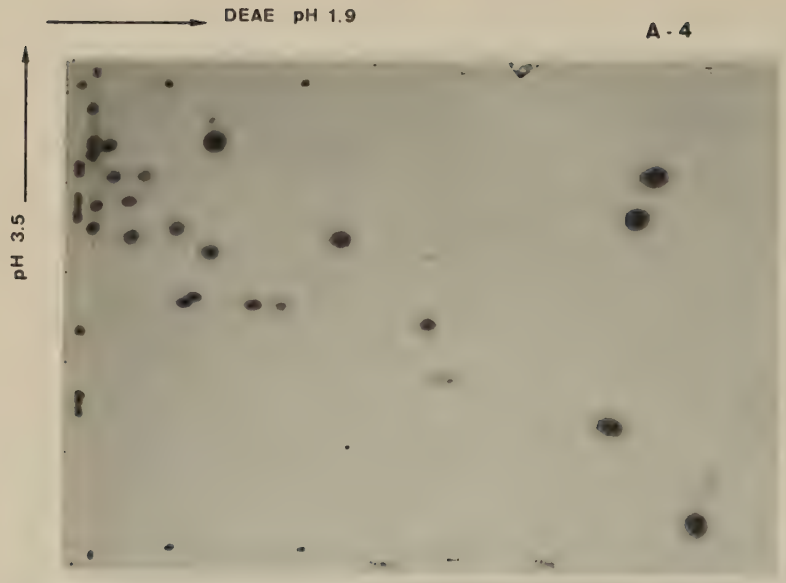


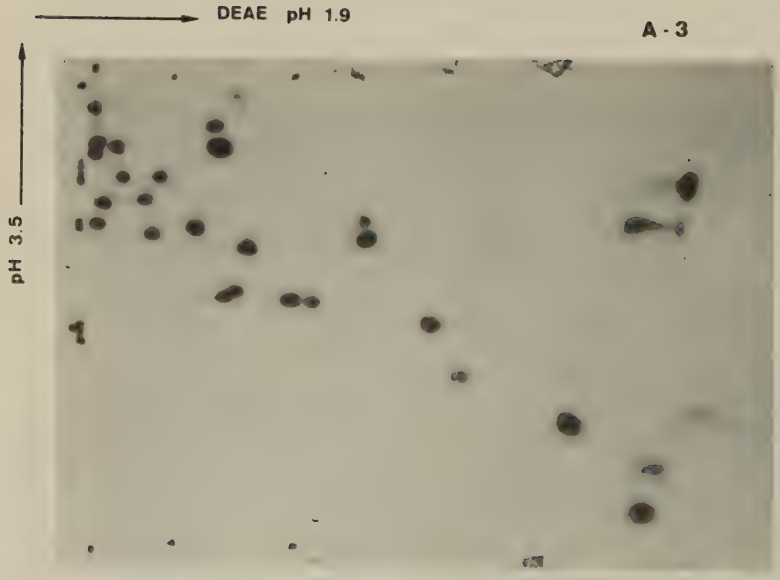

2
4
5
7
6
8
9
14
9a
11
17
15
16
20
23
26
27
28
29
30
bl
32
31
34
35
37a
37
39
38

A - 1'

2
4
6
5
7
8
9
11
14
9a
17
15
16
20
23
6
27
28
29
30
bl
32
31
34
35
37a
37
39
39

A - 2'


DEAE pH 1.9
A - 4
pH 3.5


DEAE pH 1.9
A - 3
pH 3.5

A - 4'
A - 3'

Table 3.1 RNase T_1 oligonucleotide catalog of the RNA fragment M-2a

T_1 oligonucleotide	Molar yield	Sequence
2	0.7	AUUUCAAUUUCUCACUG[G]
4	1.0	UCUCUAUAG[G]
5	0.8	UAUACACAUG[G]
6	0.6	CUUUAACG[A]
7	1.0	UUCCUG[G]
9	0.8	CUAAUAG[G]
11	0.6	AAAAAUAG[C]
14	1.0	UCAUG[U]
15	1.0	UAUG[U]
16	1.0	UUCCG[U]
17	2.2	UUG[A], UUG[C]
20	0.7	ACUACAG[U]
23	1.1	AAUG[U]
26	1.1	AUG[U]
27	4.1	CCUG[A], CUCG[U], CUCG[U], UCCG[U]
28	1.0	UAG[A]
29	1.3	CUG[A]
30	2.3	UG[A], UG[C]
31	0.8	CCAAG[C]
32	0.4	AACG[C]
34	2.0	CAG[A], CAG[C]
35	6.0	AG[A], AG[U], 3 AG[G]
37	2.6	CG[U], CG[A]
39	10.0	G[U], 2 G[A], 2 G[G], 5 G[C]

Table 3.2 RNase A oligonucleotide catalog of the RNA fragment M-2a

Panc oligonucleotide	Molar yield	Sequence
1	0.7	GAGGGU[A]
2	1.2	GAGGAU[U]
3	0.9	AGGAGAU[G]
4	0.9	AGAAAAAU[A]
5	0.9	AGAGGC[U]
6	1.1	GAGU[C]
7	0.9	GGGC[G]
8	1.0	GAAU[G]
9	1.0	AGGC[C]
10	1.1	AGU[G]
11	1.9	GGC[A], GGC[U]
12	7.4	2 GU[A], 3 GU[U], 2 GU[C], GU[G]
13	0.9	AAGC[U]
14	0.9	GAAC[G]
15	1.9	AAU[A], AAU[U]
16	1.7	AGC[A], AGC[C]
17	0.9	GAC[U]
18	6.0	2 AU[A], 3 AU[G]
19	3.3	2 GC[U], GC[G]
20	1.3	AAC[G]
21	1.0	G[C]
22	4.9	3 AC[A], AC[U]
23	13.8	3 C[C], 4 C[A], 4 C[G]
24	24.8	4 U[A], 5 U[U], 6 U[G], 8 U[C]

5'-end-UUCCUGGCAGCAGAGGCUGAAUGUGCUUUAACGAACGCUCGUGUGUAUAGGCCUGACUGCAGUGAGGAUUUCAAUUUCUCACUG

```
  U A
A     C
  U A
  G C
  U A
  A U
  C G
  U G
  G C
  A U
  G C
  C G
GG UAGAAAAAUAGCCAAGCUAAUAGGAGAUGUUCCGUCCGUUGAGGGUAUGUUGCGUCACUGCCG[A]-3'-end
```

ΔG = -9.6 kcal

Fig.3.3 Nucleotide sequence of the RNA fragment M-2b. The secondary structure was derived according to the rules of Tinoco et al. (1971)

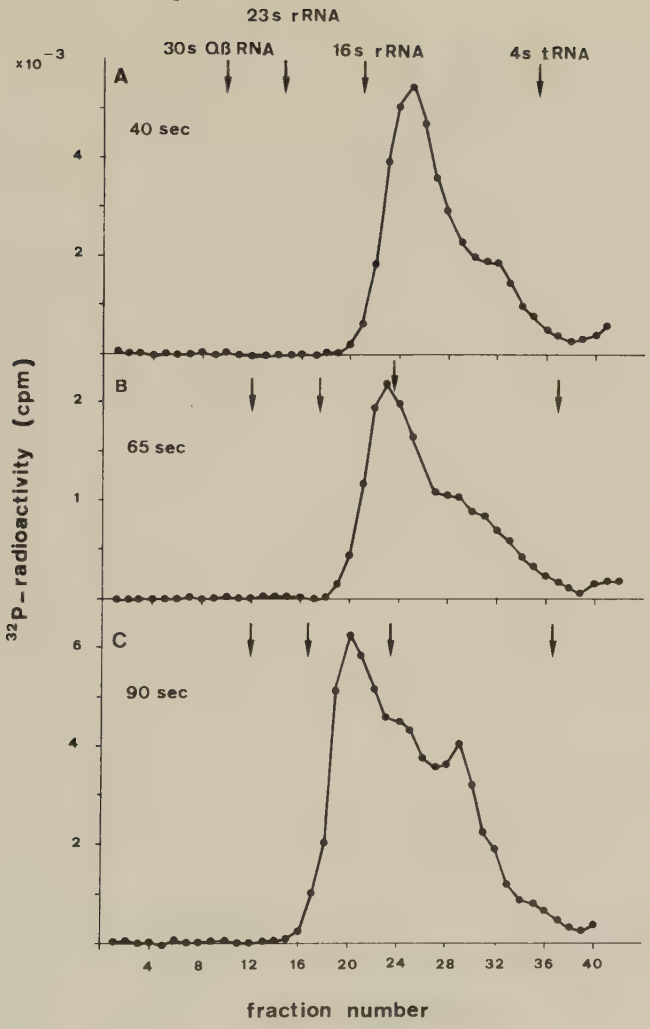
Tables 3.1 and 3.2 give the RNase T_1 and RNase A oligonucleotide catalogs of the RNA fragment M-2a. A detailed derivation of the sequence of each oligonucleotide is given in the Appendix. Using overlaps, nearest neighbour data and information obtained by partial digestion products of the fragments M-2a and M-2b (see Methods section and Appendix) the complete nucleotide sequence of these fragments could be derived. Fig. 3.3 shows the complete nucleotide sequence of the RNA fragment M-2b. Fragment M-2b contains all the sequences present in M-2a and extends the sequence of M-2a by 9 nucleotides at the 3'-end, as discussed in detail in the Appendix.
No overlap with any known sequence of Qβ RNA was found so that the RNA fragment M-2b cannot be located on the genome.
The most stable secondary structure of the RNA fragment M-2b, derived according to Tinoco et al. (1971), resulted in a structure having one basepaired region with a free energy of -9.6 kcal. This helical region represents only about 13% of the of the intact RNA fragment. This value is low compared to a high degree of secondary structure (63-82%) estimated for viral RNAs in general (Mitra et al., 1963; Boedtker, 1967; Isenberg, 1971).

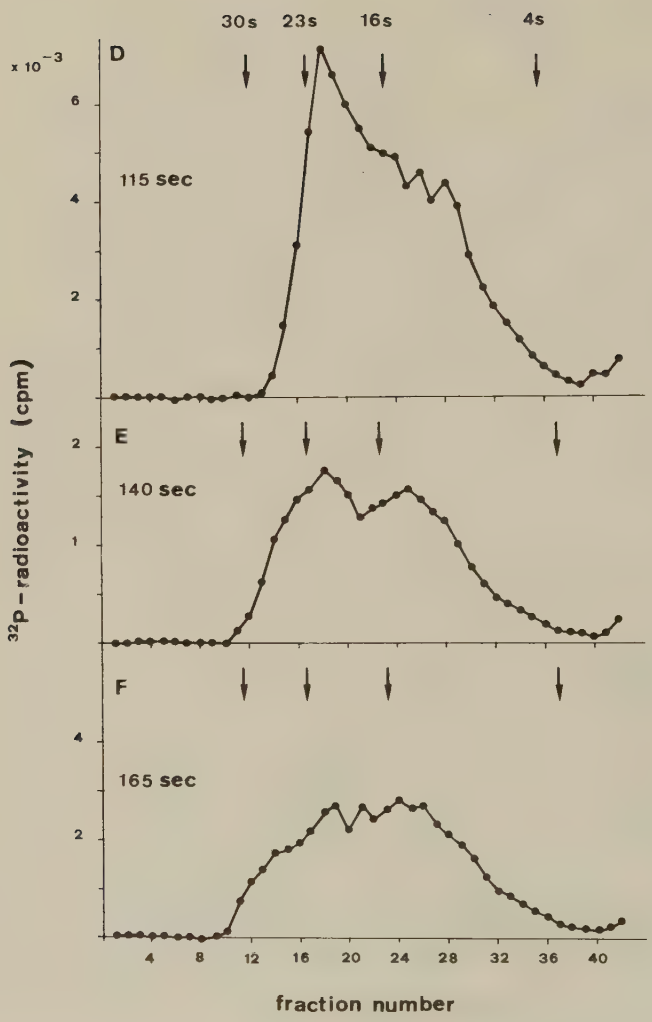
3.3. Location of the M-site on Qβ RNA

In order to determine the position of the RNA segments of the M-site on Qβ RNA, a technique similar to the one taken previously to locate the S-site and the coat cistron on Qβ RNA was used (Weber et al., 1972; Hindley et al., 1970). The experimental approach involves the in vitro synthesis of a collection of full-length Qβ RNA strands, labeled to different and known extents from the 5'-end with a ^{32}P-labeled triphosphate. Qβ replicase is bound to these RNA preparations under M-conditions and the RNA segments corresponding to the binding site are isolated and characterised. Since only molecules labeled up to or beyond the M-binding site can yield ^{32}P-labeled fragments,

Figure 3.4 Sedimentation profiles of ^{32}P-labeled Qβ RNA segments, resulting from short-time, synchronized synthesis by Qβ replicase.

Qβ replicase (200 units) was preincubated with denatured replicative intermediate (200 µg), (Weissmann et al., 1967) in 1.25 ml of a reaction mixture containing 80 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 1 mM EDTA; unlabeled GTP (0.8 mM), α-^{32}P ATP (0.15 mM, specific activity: 6.4 10^7 cpm/nmole) for 10 min at 37°C in order to get a synchronized initiation of Qβ RNA (plus strand) synthesis (Billeter et al., 1969a). Chain elongation was started by adding unlabeled CTP, GTP and UTP (final concentration: 0.8 mM) and 80 µg/ml polyethylene sulfonate, an inhibitor of chain initiation (Kondo & Weissmann, 1972).The final reaction volume was 1.5 ml. Incubation was continued at 37°C and 250 µl aliquots were removed after 40 sec (A), 65 sec (B), 90 sec (C), 115 sec (D), 140 sec (E) and 165 sec (F). Each sample was adjusted to 25 mM EDTA in order to stop synthesis, chromatographed on Sephadex G-50 (2 ml bed volume, equilibrated in 60 mM Tris-HCl (pH 7.5))at 4°C and the excluded fractions were pooled. Aliquots (10 µl) were removed and treated for 15 min at 37°C with 400 µg of pronase in the presence of 0.2% SDS and the samples were twice extracted with phenol. The RNA was precipitated with ethanol and redissolved in 200 µl H_2O containing unlabeled yeast tRNA, E. coli ribosomal RNA and full-length ^{3}H Qβ RNA. The samples were heat denatured (90 sec in boiling water) and layered on sucrose gradients (5-23%). Centrifugation was at 32'000 rpm for 14.5 h at 15°C. The peak position of the size markers are indicated by arrows.
The remainder of each sample was elongated as described in the legend to Fig. 3.5.


23s rRNA
30s QB RNA
16s rRNA
4s tRNA
x10⁻³
A
40 sec
B
65 sec
C
90 sec
32P-radioactivity (cpm)
fraction number


30s
23s
16s
4s
D
x 10⁻³
115 sec
E
140 sec
F
165 sec
32P - radioactivity (cpm)
fraction number
4
8
12
16
20
24
28
32
36
40

the site can be located.

Using Qβ minus strand as template, 6 samples of Qβ RNA, labeled to various extents from the 5'-end with ^{32}P AMP were synthesized in vitro (samples A-F).The synthesis of plus strand RNA was carried out in a synchronized fashion for different periods of time (A, 40 sec; B, 65 sec; C, 90 sec; D, 115 sec; E, 140 sec, F, 165 sec) in the presence of ^{32}P-labeled ATP. At the indicated times aliquots were removed, the RNA was purified and the sedimentation coefficients of the ^{32}P-labeled products were estimated by centrifugation through a linear sucrose gradient using internal standards as size markers (Fig. 3.4). The s-values of the ^{32}P-labeled RNA species were evaluated from a plot of s-values of the markers versus their sedimentation distance (see Table 3.3). The corresponding chain lengths were deduced from the sedimentation coefficients by using the empirical correlation: $S/S_1 = (M/M_1)^k$, with $S_1 = 30.5$; $M_1 = 4500$; $k = 0.56$ (Kurland, 1960; Studier, 1965; Boedtker, 1971). The results are summarized in Table 3.3.

With the remainder of the ^{32}P-labeled non-elongated RNA segments, synthesis was continued in the presence of unlabeled triphosphates to allow completion of the plus strand RNAs.

The six elongated preparations were centrifuged through a sucrose gradient, full size Qβ RNA was selected and Qβ replicase was bound to each sample, as well as to uniformly labeled Qβ RNA. The RNA fragments remaining bound to Qβ replicase after RNase T_1 digestion were isolated and fractionated on a polyacrylamide gel (Fig. 3.5). It can be seen that bands M-2a/b, M-5, M-11 as well as the non-analysed bands M-6, M-7, M-8 are not present in the preparations A, B, C, whereas in preparation D, they are fully labeled. As preparations C and D are derived from RNA segments which have an average chain length of about 2100 and 2600 nucleotides, respectively (Table 3.3), it can be concluded that the protected RNA fragments M-2a/b, M-5 and M-11

Table 3.3 Chain length estimation of labeled Qβ RNA segments synthesized in vitro.

Sample	Labeling time (sec)	Sedimentation coefficient: s[a]	Chain length[b] (number of nucleotides)
A	40	14	1070
B	65	17	1580
C	90	20	2070
D	115	22	2530
E	140	24	2970
F	165	24*	3000*

a) The sedimentation coefficients were evaluated from a plot of s-values of the markers, indicated by arrows in Fig. 3.4, versus their sedimentation distance. The position of the leading edge of the product peak was measured and its s-value determined by linear interpolation between two adjacent markers.

b) The chain lengths were derived from the sedimentation coefficients using the correlation, $S/S_1 = (M/M_1)^k$ with $S_1 = 30.5$, $M_1 = 4500$, $k = 0.56$.

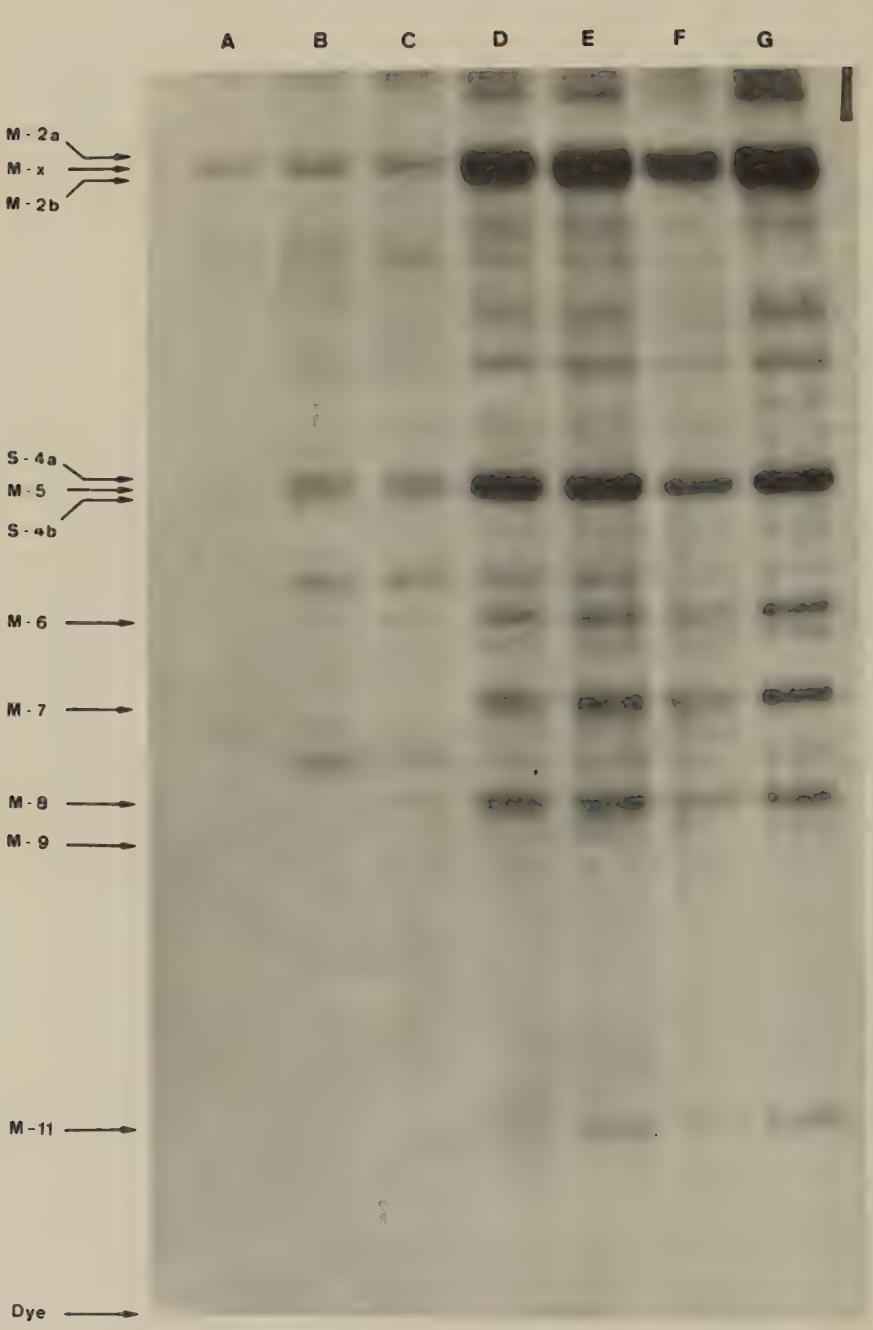
*) This value could not be evaluated any more as the RNA population was too heterogeneous in size.

Figure 3.5 Location of the position of the bound RNA segments on Qβ RNA.

^{32}P AMP-labeled Qβ RNA segments of different lengths, synthesized as described in the legend to Fig. 3.4 (Pulse fractions, samples A-F) were elongated (to allow completion of the nascent chains) in the presence of unlabeled ATP, GTP, CTP and UTP, each at a final concentration of 0.8 mM; EDTA (final concentration: 1 mM); $MgCl_2$ (final concentration: 12 mM); polyethylene sulfonate (final concentration: 80 µg/ml) by allowing synthesis to occur for 5 min at 37°C. The reaction mixtures were then adjusted to 25 mM EDTA, treated for 15 min at 37°C with 400 µg pronase in the presence of 0.5% SDS and extracted with phenol. The RNA was precipitated with ethanol and dissolved in 100 µl TNE. The solution was filtered through Sephadex G-50 columns (2 ml bed volume, equilibrated in TNE). The excluded fractions were pooled, the RNA precipitated with ethanol and dissolved in 200 µl H_2O. After heat denaturation (90 sec in boiling water), the partially labeled full-length (30 s) RNA was isolated by sedimentation through a linear sucrose gradient (5-23%).

The partially labeled Qβ RNA preparations A ($2.7\ 10^6$ cpm, 10µg), B ($2.1\ 10^6$ cpm, 6 µg), C ($3.7\ 10^6$ cpm, 7.5 µg), D ($3.3\ 10^6$ cpm, 5 µg), E ($4.5\ 10^6$ cpm, 5 µg), F ($3.9\ 10^6$ cpm, 5 µg) as well as uniformly labeled Qβ RNA used as control G ($4.6\ 10^6$ cpm, 24 µg) were bound to Qβ replicase under M-conditions, the binding complexes were digested by RNase T_1 and the bound fragments were recovered and separated on a polyacrylamide gel, as described in Methods, section 2.2.1.

Labeleling times: A, 40 sec; B, 65 sec; C 90 sec; D, 115 sec; E, 140 sec; F, 165 sec.


A
B
C
D
E
F
G
M - 2a
M - x
M - 2b
S - 4a
M - 5
S - 4b
M - 6
M - 7
M - 8
M - 9
M - 11
Dye

(as well as the non characterised fragments M-6, M-7, M-8) are located between the 2100th and 2600th nucleotide from the 5'-end of Qβ RNA, that is around the middle of the genome. Since these fragments appear clustered in a restricted area of the genome, this region is called M-site.

As can be seen in Fig. 3.5, in the vicinity of band M-5, a double band (called S_{4a} and S_{4b}) is visible in preparations B and C. Furthermore a band (called M_x) between M-2a and M-2b is visible in preparations A, B and C. It could be demonstrated that the RNA band M_x was not due to the presence of authentic M-2a/b and S_{4a}, S_{4b} were not derived from M-5 but that these bands consisted of contaminating fragments of a different origin. In order to achieve this, characteristic oligonucleotides from M-2a/b (AAAAAU, see Table 3.2) and M-5 (AAAG and AAAU, Weber, H., unpublished results) were used to monitor the appearance of these RNA segments. For this purpose the areas corresponding to the bands M-2a/b and M-5 (from the preparations A-D) were extracted and analysed by total RNase T_1 and RNase A digestion . The yield of these ^{32}P-labeled binding site oligonucleotides was plotted against the length of the ^{32}P-labeled segment of the RNA from which they are derived. The result is shown in Fig. 3.6. The band M_x present in samples A to C did not contain the characteristic oligonucleotide AAAAAU present in M-2a/b but did contain AAAG, the yield of which remained constant from A-D (E and F were not analysed). The origin of the band M_x is unknown. The bands called S_{4a} and S_{4b} are probably derived from the S-site (Fig. 3.2.B) as judged by their mobility and the oligonucleotide AAAC,released by RNase A (Weber et al., 1972). The oligonucleotide AAAC was undetectable in preparation A but was present in preparations B-D (E and F were not analysed) at a constant yield, which is in agreement with the previous finding that the S-site is located around 1200 nucleotides from the 5'-end (Weber et al., 1972) and therefore should be fully

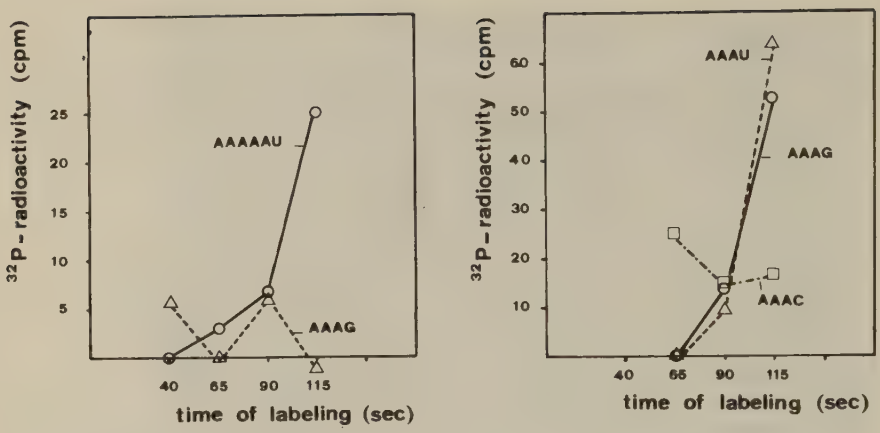


Figure 3.6 Yield of ^{32}P-labeled binding site oligonucleotides from RNA labeled to different extents.

Full-length Qβ RNA molecules, labeled to different extents from the 5'-end were bound to Qβ replicase (as described in the legend to Fig. 3.5) and the protected RNase T_1 resistant RNA fragments were fractionated on a 20% polyacrylamide gel (see Fig. 3.5). The area covering the bands M-2a, M-2b, M_x and S_{4a}, M-5, S_{4b} were extracted from the gel (see Methods) and totally digested with a mixture of RNase T_1 and RNase A. The resulting oligonucleotides were separated by electrophoresis on DEAE-paper, the radioactivity present in AAAAAU and AAAG (the region comprising M-2a/b and M_x) as well as AAAG, AAAU, AAAC (the region comprising S_{4a}, S_{4b} and M-5) was determined and is presented as a function of the time during which the RNA was labeled (40 sec = sample A; 65 sec = sample B; 90 sec = sample C; 115 sec = sample D, see Fig. 3.5).

labeled in preparation B.
The low yield of AAAC (about 20% of AAAU or AAAG) is to be expected, as under M-conditions, M-site is predominantly bound by Qβ replicase. Only about 15-25% of the bound sequences are derived from S-site (see section 3.7) under these ionic conditions.

3.4 Specific interaction of Qβ replicase with RNA fragments isolated from the binding sites

It was of interest to demonstrate that replicase is able to interact specifically with fragments from the binding sites. The capacity of ^{32}P-labeled RNA fragments M-2a/b and S-3 (see Fig. 3.2) to bind specifically to replicase was tested by binding them in presence of competing unlabeled Qβ RNA which had been randomly fragmented to the same average size (about 160 and 100 nucleotides, respectively. In a control experiment an equal amount of ^{32}P-labeled Qβ RNA, fragmented to the same size as the fragments M-2a/b or S-3, was used for binding.
Fig. 3.7.A shows that under M-conditions, if ^{32}P-labeled RNA fragment M-2a/b is competed by unlabeled randomly fragmented Qβ RNA of the same size, only 15% of the radioactivity are displaced by an equimolar amount of the unlabeled fragments, whereas the competition is at most 50% by a forty fold molar excess. In a control experiment using ^{32}P-labeled random Qβ RNA fragments, at the same ratios of unlabeled to labeled fragments, 5o% and 93% competition are obtained, respectively. Similarly, under S-conditions (Fig. 3.7.B), ^{32}P-labeled RNA fragment S-3 is only competed to about 5% at equimolar amounts and to about 20% at a 38 fold molar excess of unlabeled RNA fragments. Again ^{32}P-labeled random Qβ RNA fragments are efficiently competed out of the binding complex: at a 38 fold molar excess the competition is 84%. However in presence of

Figure 3.7 Replicase binding to the RNA fragments M-2a/b and S-3 in presence of competing random fragments.

A) Replicase binding to ^{32}P-labeled fragment M-2a/b under M-conditions in presence of random Qβ RNA fragments of the same size.

The binding mixtures contained in 7 µl: 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.2 mg/ml BSA, 2 mM DTT (binding solution); 26 pmoles/ml (1.4 µg/ml) ^{32}P-labeled fragment M-2a/b or ^{32}P-labeled randomly fragmented Qβ RNA of the same size as M-2a/b (specific activity: 2.69 10^4 cpm/pmole) (isolated by polyacrylamide gel electrophoresis as described in Methods, section 2.2.2); 167 pmoles/ml (36 µg/ml) Qβ replicase. Unlabeled randomly fragmented Qβ RNA of the size of M-2a/b (isolated by sucrose gradient centrifugation as described in Methods, section 2.2.2) were added in increasing amounts up to a 40 fold excess over the labeled RNA fragments. After 10 min incubation at 37°C, the mixtures were diluted with 0.2 ml of binding solution (without BSA and DTT) and filtered through Millipore filters. The filters were then washed 3 times with 1 ml of binding buffer (without BSA and DTT), dried and the radioactivity was counted. 100% binding = 0.07 pmoles bound per 1.15 pmoles of enzyme present. ■—■ Binding of ^{32}P M-2a/b, ●—● binding of ^{32}P -labeled randomly fragmented Qβ RNA in the presence of unlabeled randomly fragmented Qβ RNA of the same size.

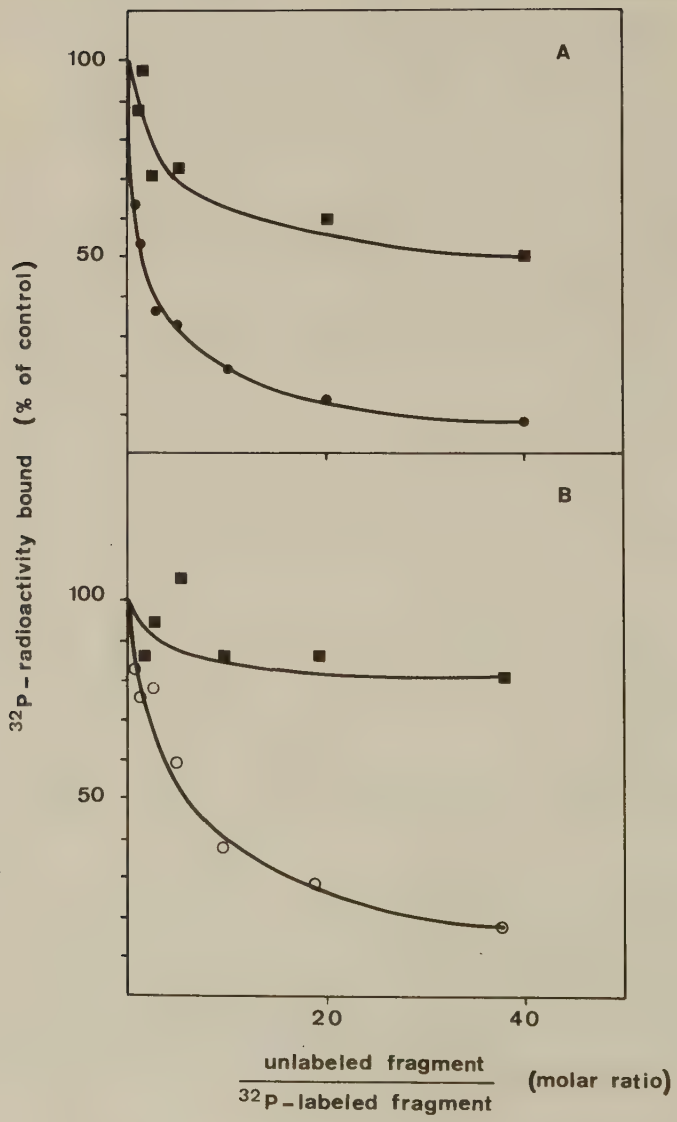
B) Replicase binding to ^{32}P-labeled fragment S-3 under S-conditions inthe presence of randomly fragmented Qβ RNA of the same size.

The binding mixture contained in 7 µl: 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.2 mg/ml BSA, 2 mM DTT (binding solution); 30 pmoles/ml (1.4 µg/ml) ^{32}P-labeled fragment S-3 or ^{32}P-labeled randomly fragmented Qβ RNA of the same size as S-3 (specific activity: 2.69 10^4cpm/pmole) isolated by polyacrylamide gel electrophoresis as described in Methods, section 2.2.2); 167 pmoles/ml (36 µg/ml) Qβ replicase. Unlabeled randomly

fragmented Qβ RNA of the size of S-3 (isolated by sucrose gradient centrifugation as described in Methods, section 2.2.2) were added in increasing amounts up to a 38 fold excess over the labeled RNA fragments. After 10 min incubation at 37°C, the reaction mixtures were diluted with 0.2 ml binding buffer (without BSA and DTT) and further processed as described in the legend to Fig. 3.7.A. 100% binding = 0.09 pmoles bound per 1.15 pmoles of enzyme present).

■—■ Binding of ^{32}P S-3, o—o binding of ^{32}P-labeled randomly fragmented Qβ RNA in the presence of unlabeled randomly fragmented Qβ RNA of the same size.

The radioactivity bound in the absence of replicase (1.0-1.5% of the amount bound in the presence of replicase) was subtracted from each point.


A
B
100
50
100
50
20
40
^{32}P - radioactivity bound (% of control)
unlabeled fragment
^{32}P - labeled fragment
(molar ratio)

equimolar amounts of unlabeled fragments only 25% of the radioactivity is desplaced instead of the 50% expected. The reason for the non-ideal isotopic dilution behaviour in the control assay under S-conditions is not clear. Nevertheless, the results of these binding experiments show unambiguously, that the binding site fragments M-2a/b and S-3 do interact in a specific way with the enzyme.

3.5 Inhibition of initiation of Qβ RNA replication by the fragments M-2a/b and S-3

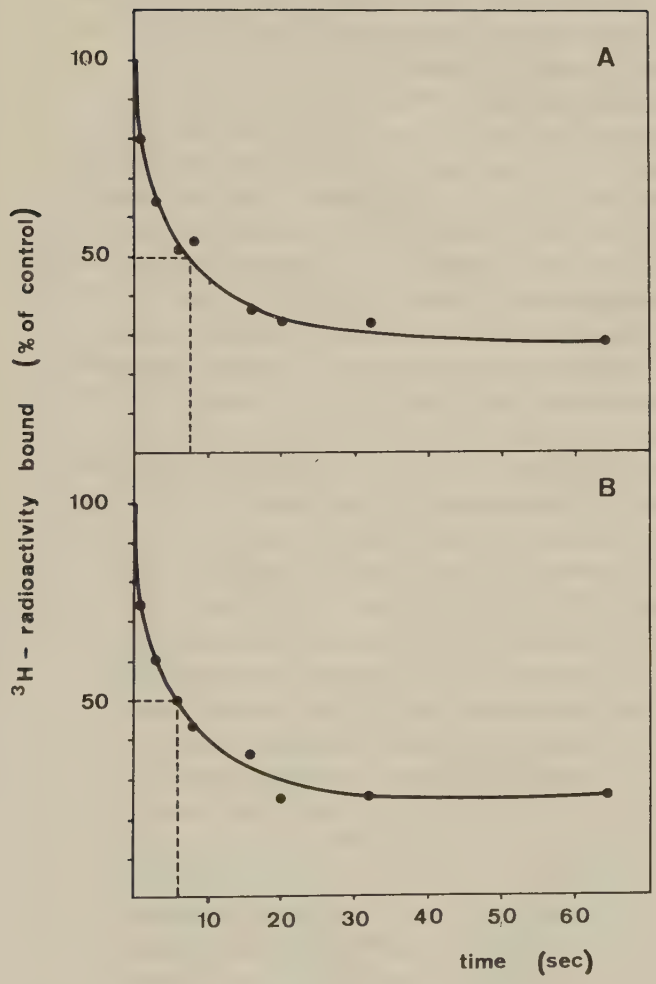
In view of the findings that 3'-terminal fragments of Qβ RNA function as replicase templates provided they are longer than 50% of the total RNA length (Schwyzer et al., 1972) and that the M-site is located around the middle of the genome (section 3.3), we suspected that M-site could be involved in template recognition. This hypothesis could now be tested, by studying the effect of the binding site RNA fragments M-2a/b and S-3 on the initiation of Qβ RNA replication. A prerequisite for the feasibility of the experiment is that the dissociation rate of the fragment/replicase binding-complex is slow enough to to allow the measurement of the possible effect of the fragments on initiation. That this is the case was shown by determining the dissociation rate of the complex between the fragments M-2a/b or S-3 and replicase in presence of excess Qβ RNA (see Fig. 3.8). 50% competition is obtained after about 8 min for M-2a/b (Fig. 3.8.a) and 6 min (Fig. 3.8.b) for S-3. 25-30% of the bound fragments appear to be irreversibly bound.
In order to determine the initiation of replication, we proceded as described by Schwyzer (1973). The assay measures the kinetics of formation of an initiation complex which is allowed to occur in presence of GTP and ATP for a number of time points. Elongation is then started by the addition of the missing ribonucleoside triphosphates, while further initiation is

Figure 3.8 Dissociation rate of the S-3 fragment-Qβ replicase and M-2a/b fragment Qβ replicase complex in the presence of excess Qβ RNA.

A solution containing 0.1 μg ^{3}H-labeled M-2a/b fragment (A) or ^{3}H-labeled S-3 fragment (B) (specific activity: $1.92\ 10^{4}$ cpm/μg); 80 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 1 mM EDTA, 0.2 mg/ml BSA, 2 mM DTT and 3.6 μg Qβ replicase in 115 μl was incubated for 9 min at 37°C. A 10 μl aliquot was removed, diluted with 200 μl washing buffer (80 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 1 mM EDTA) and filtered through a Millipore filter. The filter was washed 3 times with 1 ml of washing buffer. After 10 min of incubation 24 μg Qβ RNA in 3 μl were added and then 10 μl aliquots were removed at the indicated times. The aliquots were diluted with 200 μl washing buffer and filtered as above. The filters were dried and the radioactivity was counted as described in the legend to Fig. 3.1.

A: Dissociation of the M-2a/b fragment-replicase complex (100% binding = 0.1 pmoles bound).

B: Dissociation of the S-3 fragment-replicase complex (100% binding = 0.16 pmoles bound).

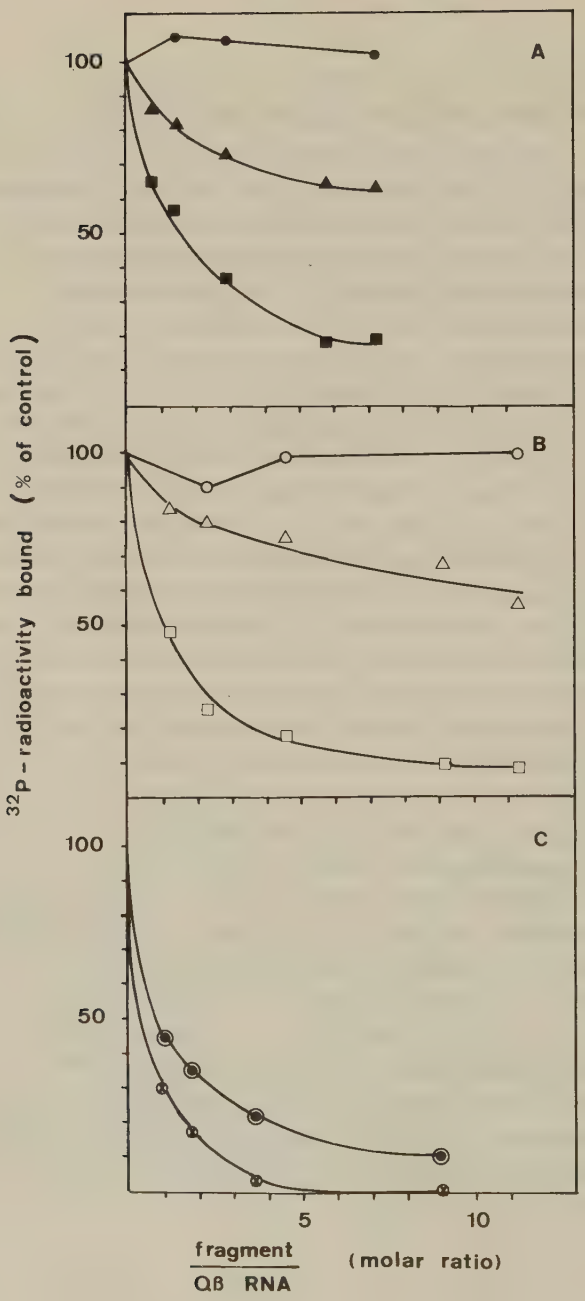

A
B
100
50
100
50
^{3}H - radioactivity bound (% of control)
10
20
30
40
50
60
time (sec)

inhibited (by the addition of polyethylene sulfonate, Kondo & Weissmann, 1972), so that only chains which had been initiated during that period of time can be further elongated.
This arrangement yields an extremely sensitive determination of initiation since each initiation event results in the synthesis of a full-size Qβ minus strand.
In the present experiment, the RNA fragments M-2a/b and S-3 were first bound to Qβ replicase and then initiation was allowed to occur for a very short period of time (20 sec) in presence of GTP and α-^{32}P ATP. Completion of the initiated chains was followed by the addition of the 2 missing ribonucleoside triphosphates (CTP and UTP) in the presence of polyethylene sulfonate. The results are shown in Fig. 3.9. It can be seen that not only fragment M-2a/b but also S-3 are inhibiting the initiation of replication: when present in amounts equimolar with Qβ RNA, inhibition is 40 and 53%, respectively. Qβ RNA fragmented to an average size of 160 and 100 nucleotides in length respectively shows a 2 to 5 times smaller inhibition. This inhibition is probably due to the sequences from M-site and S-site which are also present in the fragments. E. coli ribosomal RNA fragmented to the same size as M-2a/b or S-3 do not show any effect.

Earlier studies had shown that synthetic polynucleotides such as poly(rU), poly(rC) and to a lesser extent poly(rA) are strong inhibitors of both the plus strand and the minus strand directed synthesis (Kondo & Weissmann, 1972).
Poly(rU) and poly(rC) fragmented to an average size of about 130 nucleotides showed also a strong inhibitory effect on initiation (Fig.3.9.C).
It should be noted that all these different RNA fragments have been isolated in parallel and from the same polyacrylamide gel in order to get RNA preparations of similar size and the same degree of purity, in regard to contaminats from the gel.

Figure 3.9 Effect of the RNA fragments M-2a/b and S-3 on initiation of Qβ RNA replication.

A mixture containing 80 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 1 mM EDTA; 1 unit of Qβ replicase (specific activity: 1500 units/mg) and increasing amounts of RNA fragments (0.02-0.2 µg) in 14 µl, was pre-incubated for 3 min at 37°C. Qβ RNA directed synthesis was initiated by the addition of 10 µl of 80 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 1 mM EDTA; 0.16 mM α-^{32}P ATP (specific activity: 3 10^5 cpm/nmole) 1 mM GTP, 1 unit host factor I, and 0.8 µg Qβ RNA, which had been preincubated for 2 min at 37°C. After 20 sec, elongation was started by adding 1 µl of a solution containing 2.5 mM CTP, 2.5 mM UTP and 1.25 mg/ml polyethylene sulfonate. The reaction was stopped after 5 min and the incorporated radio-activity was determined by measuring the acid precipitable radioactivity on glass filters. The acid precipitable radioactivity incorporated in the absence of RNA fragments (100% activity) corresponded to 0.03 nmoles α-^{32}P-AMP. Acid precipitable radioactivity obtained in the absence of Qβ RNA, 1.9% from the value found in its presence, was subtracted from each point. The following RNA fragments were used: Randomly fragmented Qβ RNA of the average size of M-2a/b(▲—▲) and of the average size of S-3 (△—△), randomly fragmented E. coli ribosomal RNA of the average size of M-2a/b (●—●) and of the average size of S-3 (○—○), poly(rU) (⊗—⊗) and poly(rC) (◉—◉) having an average size of 130 nucleotides, the RNA fragments M-2a/b (■—■) and S-3 (□—□). The fragments were prepared as described in Methods, section 2.2.1 and section 2.2.2.


A
B
C
100
50
100
50
100
50
^{32}P - radioactivity bound (% of control)
5
10
fragment / Qβ RNA
(molar ratio)

The inhibitory effect of these RNA fragments is thus not due to different amounts of extraneous substances present in the preparations. This is further supported by the finding that an extract of a gel piece, devoid of RNA, did not effect the reaction (data not shown).

It was also of interest to know whether the fragments M-2a/b and S-3 would interfere with the poly(rC)-directed synthesis. If these fragments inhibit initiation by binding to the enzyme site or sites involved in substrate binding and/or the condensation reaction, one would expect them to inhibit the poly(rC)-directed synthesis as well. If their binding however occurs at another site on the enzyme which does not interfere with chain initiation and elongation per se, one might expect the fragments not to show an effect on the poly(rC)-directed synthesis.

The various fragments were incubated with Qβ replicase without substrates and poly(rC) was added subsequently to allow synthesis to occur for 2 min in presence of ^{3}H GTP. At a molar ratio of fragment over template of 5 (for M-2a/b) or 8 (for S-3) at which Qβ RNA-directed synthesis was inhibited to 80% (M-2a/b) or 90% (S-3), the poly(rC)-directed synthesis was only reduced 11 and 27%, respectively (Table 3.4). Poly(rU) however inhibited the reaction completely. From these results it can be concluded that the RNA fragments M-2a/b and S-3 specifically inhibit the formation of a productive initiation complex of Qβ RNA and replicase, by preventing Qβ RNA from binding correctly to the enzyme, whereas poly(rC) appears not to require the same recognition mechanisms.

Table 3.4

A mixture containing in 15 µl 80 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 1 mM EDTA, 0.5 units Qβ replicase (specific activity: 1500 units/mg) and various amounts of RNA fragments (as indicated) was incubated for 3 min at 37°C. Poly(rC)-directed synthesis was allowed to occur by adding 10 µl of 80 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 1 mM EDTA, 3 µg/ml poly(rC) (number average molecular weight: 10^5) and 0.68 mM ^{3}H GTP (specific activity: 6.4 10^5 cpm/nmole), which had been pre-incubated for 2 min at 37°C. The reaction was stopped after 2 min and the incorporated radioactivity was determined by measuring the acid precipitable radioactivity on glass filters. Acid precipitable radioactivity obtained in the absence of replicase, 0.4 pmoles was subtracted from each point.

Abbreviations:

rQ/M = randomly fragmented Qβ RNA of the average size of M-2a/b

rQ/S = " " " " " " " " " S-3

rR/M = randomly fragmented E. coli ribosomal RNA of the average size of M-2a/b.

rR/S = randomly fragmented E. coli ribosomal RNA of the average size of S-3.

Poly(rU): average size: 130 ± 20 nucleotides.

The RNA fragments and the homopolymers have been prepared as described in Methods, section 2.2.2.

Table 3.4 Effect of the RNA fragments M-2a/b and S-3 on poly (rC)-directed synthesis by Qβ replicase.

Fragment	Molar ratio fragm./poly(rC)	^{3}H GMP incorporated pmoles	%
none	-	28.1	100
M-2a/b	2.5	24.1	86
	5	25.1	89
S-3	4	21.6	77
	8	20.6	73
rQ/M	2.5	22.1	79
	5	24.6	88
rQ/S	4	35.6	127
	8	21.6	75
poly(rU)	3	0.0	0
	6	0.0	0
rR/M	2.5	22.6	80
	5	22.1	79
rR/S	4	24.6	88
	8	20.1	72

3.6 Binding competition between the fragments M-2a/b, S-3, poly(rU) and poly(rC)

Since both RNA fragments M-2a/b and S-3 were found to inhibit initiation of QβRNA replication (although the S site had been shown not to be essential for template recognition, Schwyzer, 1972), it was of interest to test whether these fragments do interact at one and the same site or at two different sites on the enzyme. For this purpose, binding competition assays between the various RNA fragments to the enzyme were performed.

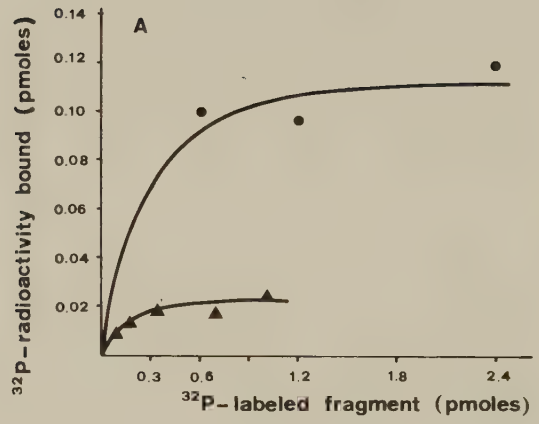
The association curves of replicase with radioactively labeled fragments M-2a/b or S-3 were studied in order to determine the ratio of fragment to enzyme at which saturation of the fragment binding sites on replicase is reached (Fig. 3.10.A). It can be seen that at saturation 0.02 pmoles M-2a/b, or 0.1 pmole S-3, are bound per 0.9 pmoles of replicase. The difference in the binding efficiencies of the two fragments was found only with the RNA preparation used for this experiment. In other prepara-preparations, under identical ionic conditions, the binding efficiencies of the 2 RNA fragments were similar (see, for instance, Fig. 3.8). It is possible that the RNA fragment M-2a/b was partially degraded and therefore had a reduced binding efficiency compared to S-3. The binding efficiency of these fragments could be increased by changing the filtering procedure as described by Hinkle & Chamberlin (1972), (see Fig. 3.10.B). As all the binding studies described so far had been performed essentially as indicated in the legend to Fig. 3.10.A, the binding studies were carried out as described there. When however binding competition experiments were done using the filtering procedure described in the legend to Fig. 3.10.B, qualitatively similar results were obtained.

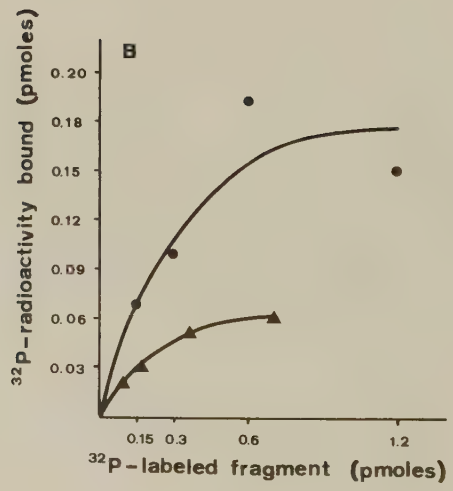
The binding curves of ^{32}P-labeled RNA fragment M-2a/b to

Figure 3.10 Replicase binding to ^{32}P-labeled RNA fragments M-2a/b and S-3.

A: Qβ replicase (0.22 μg, 1.0 pmole) was incubated for 10 min at 37°C with the indicated amounts of ^{32}P-labeled fragment M-2a/b (specific activity: 6.6 10^4 cpm/pmole) (▲—▲) or S-3 (specific activity: 2 10^4 cpm/pmole) (●—●) in a buffer containing 80 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 1 mM EDTA, 0.2 mg/ml BSA, 2 mM DTT in 10 μl. The reaction mixtures were then chilled, 0.2 ml of washing buffer (80 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 1 mM EDTA) were added and the mixtures were filtered through Millipore filters, which had been preincubated in washing buffer containing 0.5 mg/ml yeast carrier RNA to reduce free RNA binding to the filter. The filters were washed 3 times with 1 ml of washing buffer, dried and the radioactivity was measured as described in the legend to Fig. 3.1. The radioactivity retained on the filter without replicase (0.08-0.14% of the input radioactivity) was subtracted from each point.

B: as described under A, except that a different filtering procedure was used. The binding mixtures were diluted into 1 ml of cold washing buffer and filtered immediately through Millipore filters without further washing of the filters. The radioactivity retained on the filters without replicase (2-6% of the input radioactivity) was subtracted from each point.


A
0.14
0.12
0.10
0.08
0.06
0.04
0.02
^{32}P-radioactivity bound (pmoles)
0.3
0.6
1.2
1.8
2.4
^{32}P-labeled fragment (pmoles)

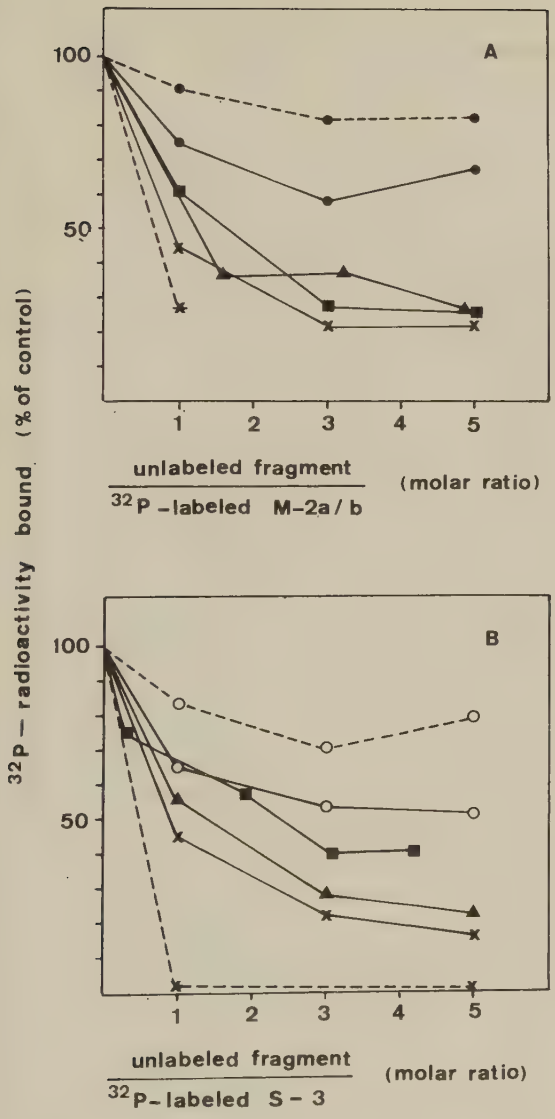

B
0.20
0.18
0.15
0.12
0.09
0.06
0.03
^{32}P-radioactivity bound (pmoles)
0.15
0.3
0.6
1.2
^{32}P-labeled fragment (pmoles)

replicase at saturating fragment to enzyme ratios (according to Fig. 3.10.A) in presence of increasing amounts of unlabeled fragment S-3 is shown in Fig. 3.11.A. Yeast carrier RNA was included in the binding mixtures (5 µg carrier RNA per µg ^{32}P-labeled RNA fragment)in order to reduce unspecific binding: 18-20% of the bound radioactivity was desplaced by yeast carrier RNA (data not shown). The results of the converse experiment in which ^{32}P fragment S-3 is competed by unlabeled M-2a/b are given in Fig. 3.11.B. The curves indicate that the S-and M-site RNA fragments do show a specific mutual competition in their binding to replicase, in contrast to ribosomal RNA fragments which do not significantly interfere with their binding. The results suggest that M-2a/b and S-3 are interacting at either the same binding site(s) on the enzyme or if there are 2 specific binding sites for the two fragments on the enzyme, that their binding is mutually exclusive. In agreement with this concept is the finding that additivity in the binding of the two specific RNA fragments to the enzyme could not be demonstrated upon simultaneously binding ^{32}P-labeled fragments M-2a/b and S-3 (data not shown).

In addition, poly(rU) and poly(rC) do compete with the binding of the M-and S-site RNA fragments (Fig. 3.11.B), showing that they have an affinity for at least two binding sites on Qβ replicase: an initiation site and the M-and/or S-site. Randomly fragmented Qβ RNA show less but significant competition probably because of the M-and S-site RNA sequences present in these RNA populations.

Figure 3.11 Replicase binding to ^{32}P-labeled fragments M-2a/b or S-3 in the presence of unlabeled fragments M-2a/b or S-3.

A: The binding mixtures contained in 10 µl: 80 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 1 mM EDTA, 0.2 mg/ml BSA, 2 mM DTT (binding solution); 2 µg/ml (38 pmoles/ml) ^{32}P-labeled fragment M-2a/b (specific activity: 6.6 10^4 cpm/pmole); 22.5 µg/ml (104 pmoles/ml) Qβ replicase; 10 µg/ml yeast carrier RNA to reduce unspecific binding and increasing amounts of unlabeled fragments as indicated in Fig.3.11.
B: The binding mixtures contained in 10 µl: 80 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 1 mM EDTA, 0.2 mg/ml BSA, 2 mM DTT (binding solution); 2 µg/ml (60 pmoles/ml) ^{32}P-labeled fragment S-3 (specific activity: 2 10^4 cpm/pmole); 22.5 µg/ml (104 pmoles/ml) Qβ replicase; 10 µg/ml yeast carrier RNA and increasing amounts of unlabeled fragments as indicated in Fig.3.11.
The binding mixtures A and B were incubated and filtered as described in the legend to Fig.3.10.A. (100% binding = 0.02 pmoles ^{32}P M-2a/b bound per 1.0 pmole of enzyme, Fig.3.11.A, and 0.1 pmoles ^{32}P S-3 bound per 1.0 pmole of enzyme, Fig. 3.11.B)
The radioactivity retained on the filter without replicase (0.2-0.26% of the input radioactivity) was subtracted from each point.
The following unlabeled RNA fragments were used: randomly fragmented E. coli ribosomal RNA of the average size of M-2a/b (●---●) or average size of S-3 (○---○), randomly fragmented Qβ RNA of the average size of M-2a/b (●—●) or average size of S-3 (○—○), poly(rU) (✖---✖) and poly(rC) (✖—✖) which have an average size of 130 nucleotides. The fragments M-2a/b (■—■) and the fragment S-3 (▲—▲).


A
100
50
1
2
3
4
5
unlabeled fragment
(molar ratio)
32P-labeled M-2a/b
B
100
50
1
2
3
4
5
unlabeled fragment
(molar ratio)
32P-labeled S-3
32P – radioactivity bound (% of control)

3.7. Binding of ^{32}P-labeled Qβ RNA to replicase under initiation conditions in the presence of the RNA fragments M-2a/b, S-3, homopolymers and polyethylene sulfonate

The binding studies of Qβ replicase to Qβ RNA described in section 3.1. were carried out in the absence of host factor I and GTP, both of which are required for initiation of replication. Under those conditions, replicase was found to bind to internal regions on Qβ RNA (M-site and S-site) but none of the prominent bands of the binding pattern contained the 3'-terminus (see Fig. 3.2). As initiation occurs exclusively at the 3'-end of the template (Billeter et al., 1969a), these findings suggested that the early events in the enzyme-template interaction do not involve binding of the 3'-end. In order to gain further information on the interacting sites on Qβ RNA we analysed the whole collection of RNA segments bound to the filter after RNase T_1 degradation of replicase-Qβ RNA complexes for the presence of large T_1 oligonucleotides. Structure and location of these oligonucleotides on the Qβ genome has been determined by Billeter, M.A., unpublished results. The T_1 oligonucleotide map (Fig. 3.12 and Table 3.5) provided us with a sensitive way of ascertaining, which regions of Qβ RNA interact with the enzyme, inasmuch as they contain a large T_1 oligonucleotide. Thus, the fragment S-3 from the S-site (Fig. 3.2.A) contains the large T_1 oligonucleotide T4a (Fig. 3.12). Furthermore the M-site RNA fragments M-2a/b and M-5 (Fig. 3.2.B) contain the large T_1 oligonucleotides T11 and T10a, respectively. The oligonucleotide T18 is the 3'-terminal oligonucleotide of Qβ RNA.

Location ot the large T_1 oligonucleotides on Qβ RNA

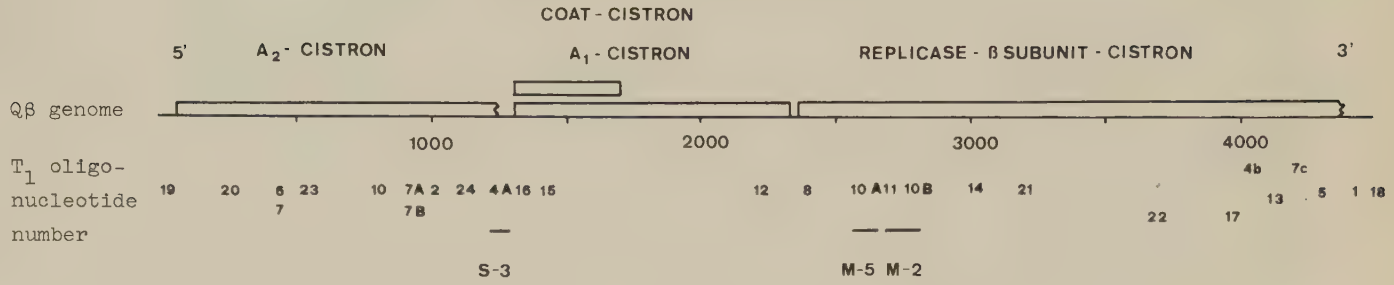


Fig. 3.12. Approximate position of the large T_1 oligonucleotides on Qβ RNA.
The figure is taken from Billeter, M.A., unpublished results.

Table 3.5. Nucleotide sequence of large T_1 oligonucleotides of Qβ RNA (Billeter, M.A., unpublished results).

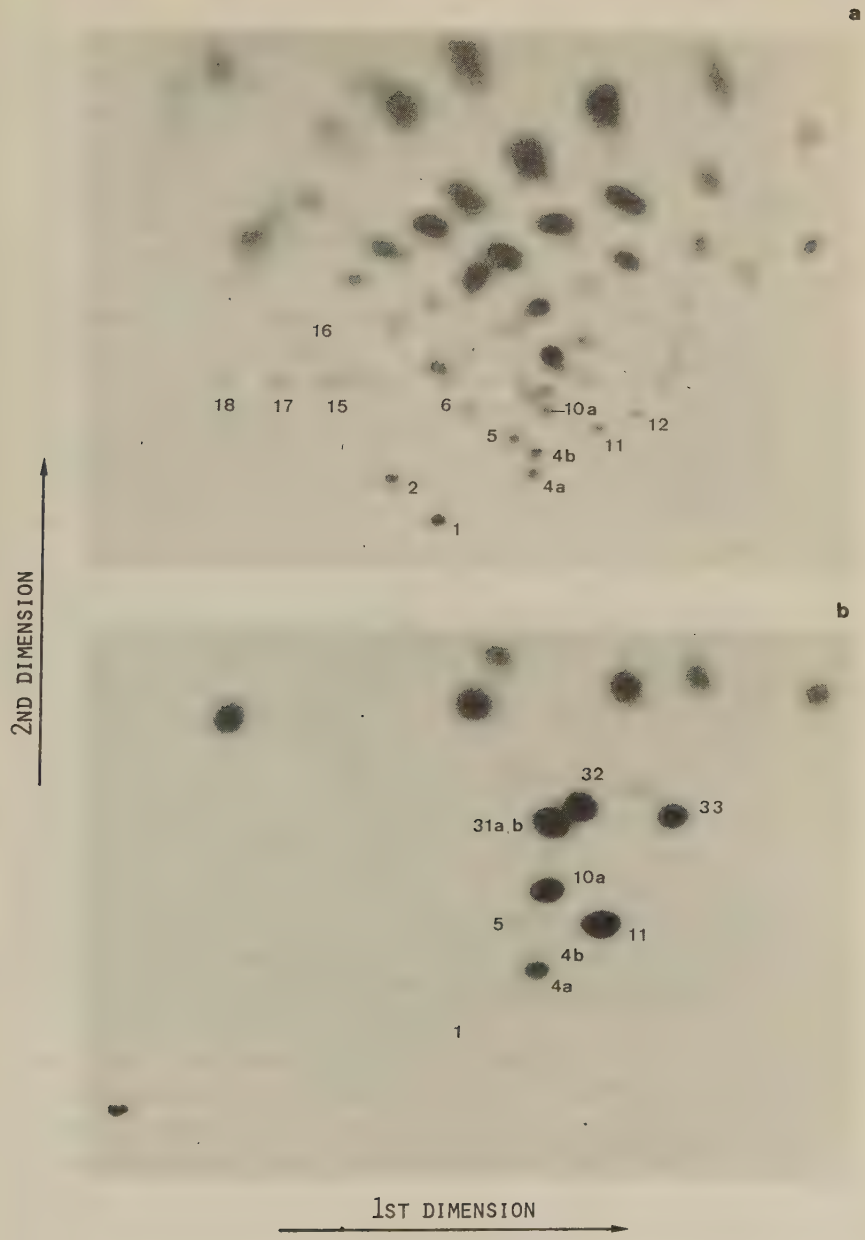
Designation	Sequence
T1	AAUAAAUUAUCACAAUUACUCUUACG[A]
T2	CUCAAAUCUUUCACCAUAAAAG[G]
T4a	UAUCUUUUUAUUAACCCAACG[C]
T4b	UUACUCCCUUUUACAUACG[U]
T5	UCCUAAUCAAUCCUUUCG[C]
T6	ACUAUAAAAUAUCUCG[G]
T7	UCCUACCAUAAUG[G]
T7a	CAUUCAUCAAUCCG[C]
T7b	UUAACUCCCUACAG
T7c	AAUAACCUAUAUCG
T8	UAACUCUCUCAG[C]
T9	UAAUACUAUACCUG[A]
T10a	AUAAAUUAUCUUAAAG[C]
T10b	CUUCUACACAUUUCG[A]
T11	AUUUCAAUUUCUCACUG[G]
T12	AUUCAUUUAUCUUAAG[U]
T13	AUUUAAUACUG[G]
T14	AUUUCUUCUAUG[G]
T15	UAAAUCCCACUAACG[G]
T16	AAAACAAACUCUG[G]
T17	ACCAAUACUAAAAAG[A]
T18	CCCUCUCUCCUCCCA$_{OH}$
T19	UCACCUCACACAG[C]
T20	CACUCCACAUCG[C]
T21	AUUCUAUAUCUCUUG[C]
T22	AUAUUAUUUUACCG[U]
T23	CCUCUCUUUUAUG[A]
T24	AUUUAUUUUACAG[C]

3.7.1 Replicase binding to ^{32}P-labeled Qβ RNA under different ionic conditions

Qβ replicase was bound to radioactively labeled Qβ RNA under various ionic conditions, the resulting binding complexes were digested by RNase T_1 in the presence of excess unlabeled Qβ RNA, the retained RNA fragments were isolated and completely digested by RNase T_1. The resulting T_1 oligonucleotides were resolved by 2 dimensional polyacrylamide gel electrophoresis. Autoradiograms of the RNase T_1 fingerprints obtained from ^{32}P-labeled Qβ RNA fragments bound to replicase under M-conditions (b), S-conditions (c) and initiation conditions (d) are shown in Fig. 3.13. Fig. 3.13.a shows the RNase T_1 fingerprint of authentic ^{32}P-labeled Qβ RNA. The relative molar yields of the ^{32}P-labeled T_1 oligonucleotides, plotted against their relative position on Qβ RNA are indicated in Fig. 3.14.
The results can be summarized as follows: The interaction between replicase and Qβ RNA involves three main regions on the genome: two internal sites (M-site and S-site) and the 3'-terminal region. The S-site was quite selectively bound under S-conditions (Fig. 3.14.b). In the presence of Mg^{2+}-ions, but in the absence of salt, M-site oligonucleotides were predominantly retained (Fig. 3.14.a,c) as reflected by the ratios of molar yields of T4a to T11 of 0.25 (Fig. 3.14.a) and 0.29 (Fig. 3.14.c), respectively.
Three major oligonucleotides 31b, 32 and 33, which had not been described before were retained under conditions where Mg^{2+} or Mn^{2+} (see below) were present. These oligonucleotides, the complete nucleotide sequence of which was not established, have been arbitrarly located in the M-site. However it is possible that these oligonucleotides are derived from different regions of Qβ RNA.
Under S- conditions (Fig. 3.14.b), the oligonucleotide designated

Figure 3.13 Two-dimensional polyacrylamide gel electrophoretic analysis of the T_1 oligonucleotides, obtained from ^{32}P-labeled Qβ RNA fragments bound to Qβ replicase, after digestion by RNase T_1.

Replicase binding to ^{32}P-labeled Qβ RNA was performed as described in the legend to Fig. 3.14. The bound RNA fragments were isolated, completely digested by RNase T_1 and the resulting oligonucleotides were fractionated by two-dimensional polyacrylamide gel electrophoresis, as described in Methods, section 2.2.1.B.
Autoradiogram of the T_1 digest, obtained from binding Qβ replicase to Qβ RNA under M-conditions (b), S-conditions (c) and I-conditions in the presence of host factor and GTP (d). (a): Autoradiogram of the T_1 digest of Qβ RNA (1 μg of ^{32}P-labeled Qβ RNA (specific activity: 3.8 10^6 cpm/μg) and 20 μg Yeast carrier RNA were digested with 10 units of RNase T_1 in 20 mM Tris-HCl (pH 7.5), 2 mM EDTA (final volume: 8μl) for 30 min at 37°C. Then 8 μl of a solution containing 500 mg of sucrose, 300 mg of urea, 2 mg of xylene cyanol FF and 2 mg of bromophenol blue per ml were added and the digest was fractionated as described above).


a
16
18
17
15
6
10a
12
5
11
4b
2
4a
1
b
32
31a,b
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10a
5
11
4b
4a
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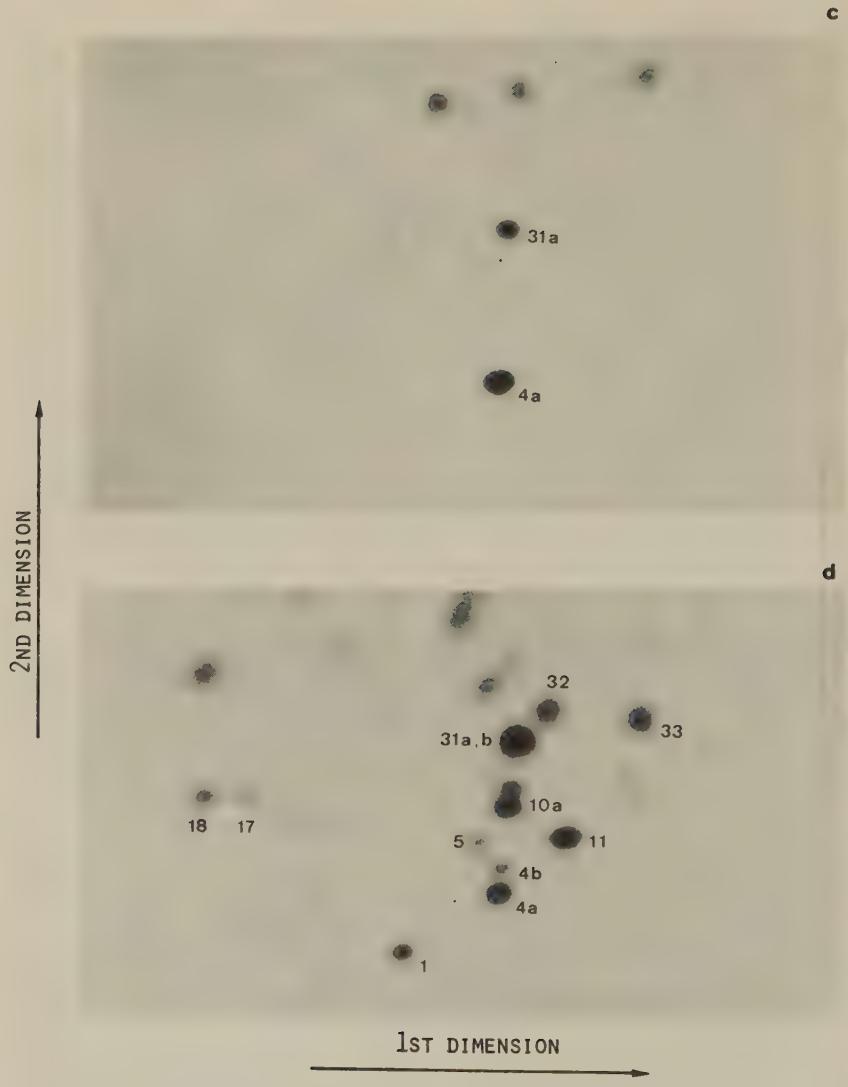
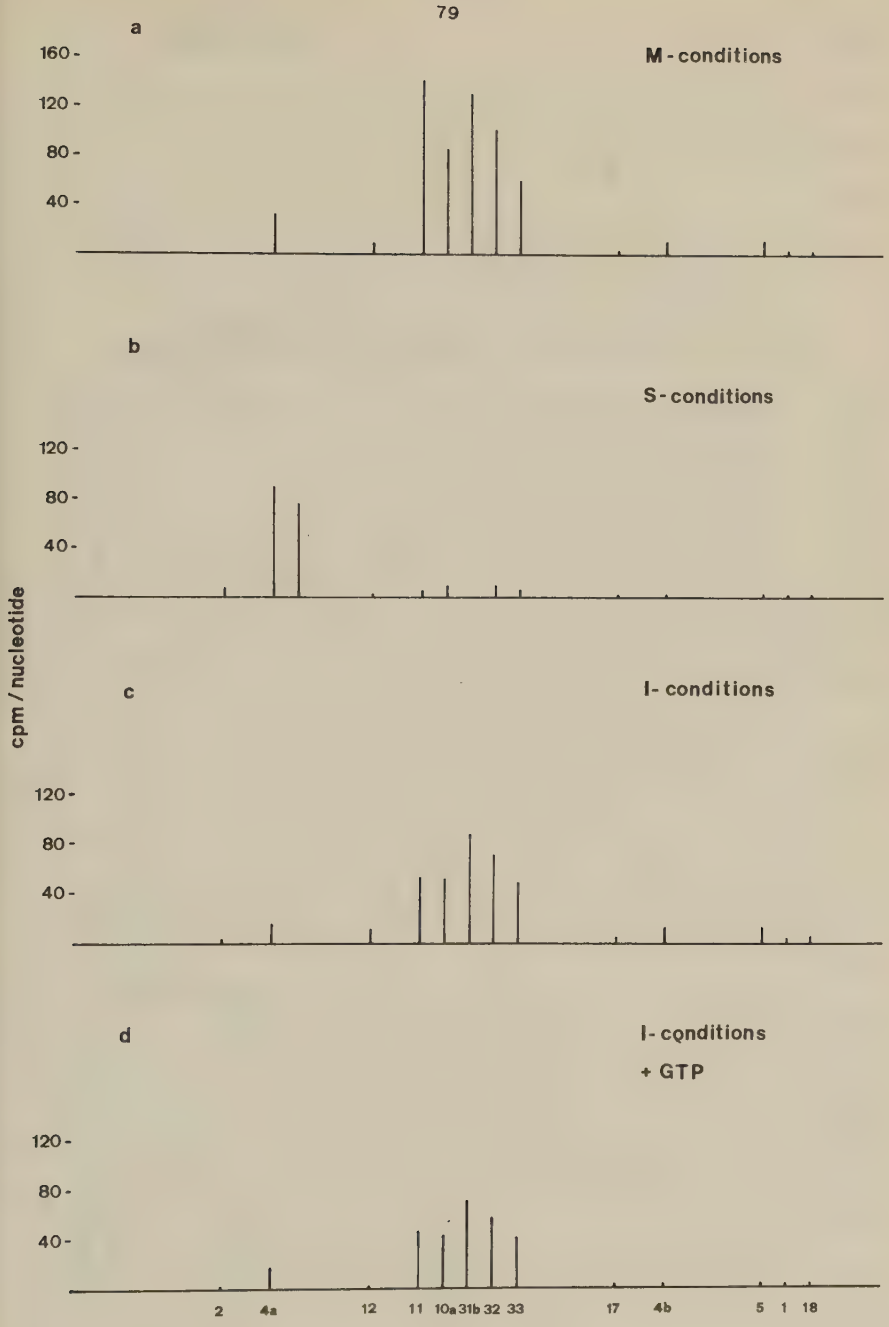
1ST DIMENSION


c
31a
4a
2ND DIMENSION
d
32
33
31a,b
10a
18
17
5
11
4b
4a
1
1ST DIMENSION

31a had a partial sequence consistent with AUACUACCUUUAG. This oligonucleotide is probably derived from the RNA fragment S-3 (see Fig. 3.2.B and Weber et al., 1972).
Addition of either GTP or host factor I alone to the binding mixture does not result in a significant modification in the binding pattern (compare Fig. 3.14.d,e to Fig. 3.14.c). However performing the binding under initiation conditions (in the presence of both host factor I and GTP) demonstrated interactions at 3 regions on Qβ RNA (Fig. 3.14.f and Fig. 3.13.d): M-site, S-site and the 3'-terminus. The ratio of molar yield of T4a to T11 was 0.62 and the ratio of T18 to T11 0.45. Under initiation conditions in the presence of host factor I, GTP and ATP essentially the same binding pattern was obtained as in Fig. 3.14.f (data not shown). The results suggest that the formation of an initiation-complex involves interactions at internal regions on Qβ RNA (M-site, S-site), which do not require GTP and host factor I and the 3'-end region, an interaction which requires host factor I and GTP. It cannot be distinguished from these data whether the 3'-end binding requires synthesis of the 5'-end of the minus strand or whether GTP binding at the initiation site is sufficient for the 3'-end binding.
It has been shown previously that by substituting Mn^{2+}-ions for Mg^{2+}-ions in an in vitro reaction, Qβ replicase accepts Qβ RNA and a variety of other RNA molecules as a template to synthesize a complementary strand. This reaction did not require host factor I (Palmenberg and Kaesberg, 1974; Obinata et al., 1975).It was of interest to analyse by using the same assay as described above, how the binding pattern was under these ionic conditions.
Under conditions where Mn^{2+}-ions were present (in the absence of Mg^{2+} and salt), replicase showed interactions with the internal binding sites (M-site and S-site) as well as with the 3'-terminal region (Fig. 3.14.g) with a 3-4 fold higher

Figure 3.14 Molar yields of ^{32}P-labeled T_1 oligonucleotides, obtained from ^{32}P-labeled Qβ RNA fragments bound to Qβ replicase after digestion by RNase T_1, plotted against their position on Qβ RNA

Replicase binding to ^{32}P-labeled Qβ RNA was performed as described in Methods (section 2.2.1) with the following specifications: 9.2 µg (a-f) or 46 µg (g,h) uniformly ^{32}P-labeled Qβ RNA (specific activity: $3.8\ 10^6$ cpm/µg (a-f), $3.5\ 10^6$ cpm/µg (g,h)) were incubated with 4.5 µg (a-f) or 22.5 µg (g,h) replicase in a 100 µl (a-f) or 500 µl (g,h) reaction volume using the following ionic conditions: M-conditions (a), S-conditions (b), I-conditions (c), I-conditions in the presence of 0.4 mM GTP (d), I-conditions in the presence of 10 units host factor I (e), I-conditions in the presence of 10 units host factor I and 0.4 mM GTP (f), Mn^{2+}-conditions (g), Mn^{2+}-conditions in the presence of 0.4 mM GTP (h); the binding mixtures were diluted into 500 µl (a-f) or 2.5 ml (g,h) of the corresponding buffers containing 45 µg (a-f) or 250 µg (g,h) of unlabeled Qβ RNA and digested as described in Methods. The bound RNA fragments were isolated and completely digested by RNase T_1. The resulting oligonucleotides were fractionated by 2 dimensional polyacrylamide gel electrophoresis and quantitated as described in Methods.
The relative molar yields are expressed in cpm per nucleotide for each oligonucleotide.


a
M - conditions
160 -
120 -
80 -
40 -
b
S - conditions
120 -
80 -
40 -
cpm / nucleotide
c
I - conditions
120 -
80 -
40 -
d
I - conditions
+ GTP
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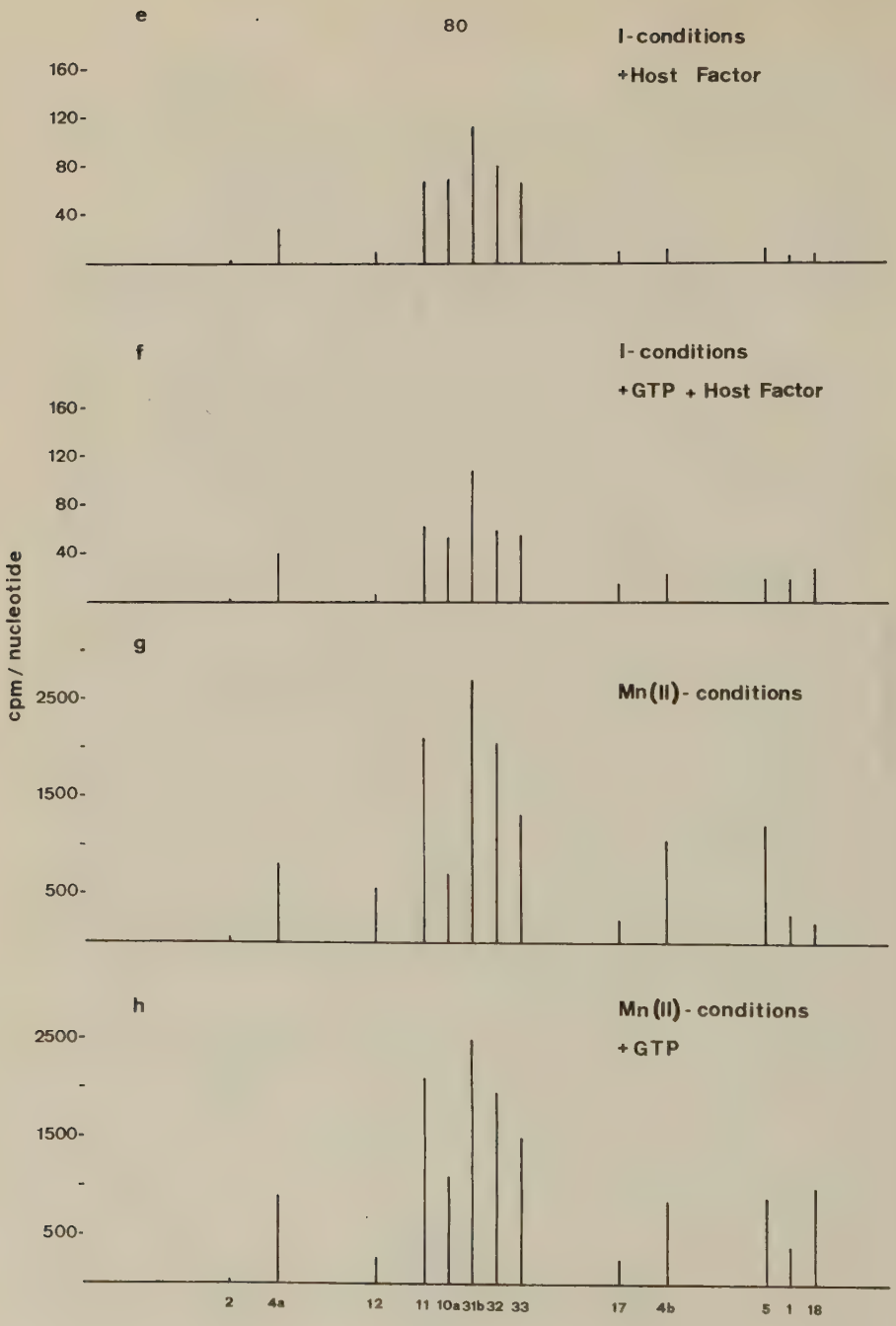
4b
5
1
18


e
80
I-conditions
+Host Factor
160-
120-
80-
40-
f
I-conditions
+GTP + Host Factor
160-
120-
80-
40-
cpm / nucleotide
g
Mn(II)- conditions
2500-
1500-
500-
h
Mn(II)- conditions
+GTP
2500-
1500-
500-
2
4a
12
11
10a
31b
32
33
17
4b
5
1
18

affinity compared to conditions where Mg^{2+} was present (Fig. 3.14.a,c,d,e,f). Addition of GTP increased the binding of the 3'-terminal oligonucleotide to replicase about 4 fold (Fig. 3.14.h).

3.7.2 Replicase binding to ^{32}P-labeled Qβ RNA in the presence of the RNA fragments M-2a/b, S-3, the homopolymers poly(rC), poly(rU) and polyethylene sulfonate

The results from section 3.5 showed that the RNA fragments M-2a/b and S-3 as well as poly(rU) and poly(rC) inhibit the initiation of replication of Qβ RNA. It was of interest to investigate how these fragments affect the binding of Qβ RNA under conditions where they inhibit initiation. Again the T_1 oligonucleotide mapping assay was used in order to identify the bound RNA segments. Polyethylene sulfonate, a known inhibitor of initiation (Kondo and Weissmann, 1972) was also included in the experiment. The unlabeled polynucleotides (or polyethylene sulfonate) were prebound to Qβ replicase and subsequently initiation was allowed to occur for a very short time period (20 sec) by adding ^{32}P-labeled Qβ RNA, host factor I and GTP. The binding mixtures were digested by RNase T_1 in the presence of excess unlabeled Qβ RNA. The bound ^{32}P-labeled RNA fragments were isolated, completely digested by RNase T_1 and the resulting T_1 oligonucleotides were resolved by 2 dimensional polyacrylamide gel electrophoresis as in section 3.7.1. The results are shown in Fig. 3.15.

The specific RNA fragments M-2a/b and S-3, which produce about a 70% reduction of the binding of intact Qβ RNA to Millipore filters prior to nuclease degradation, do not show a striking competition with specific regions of the ^{32}P-labeled Qβ RNA after digestion of the complexes by RNase T_1.

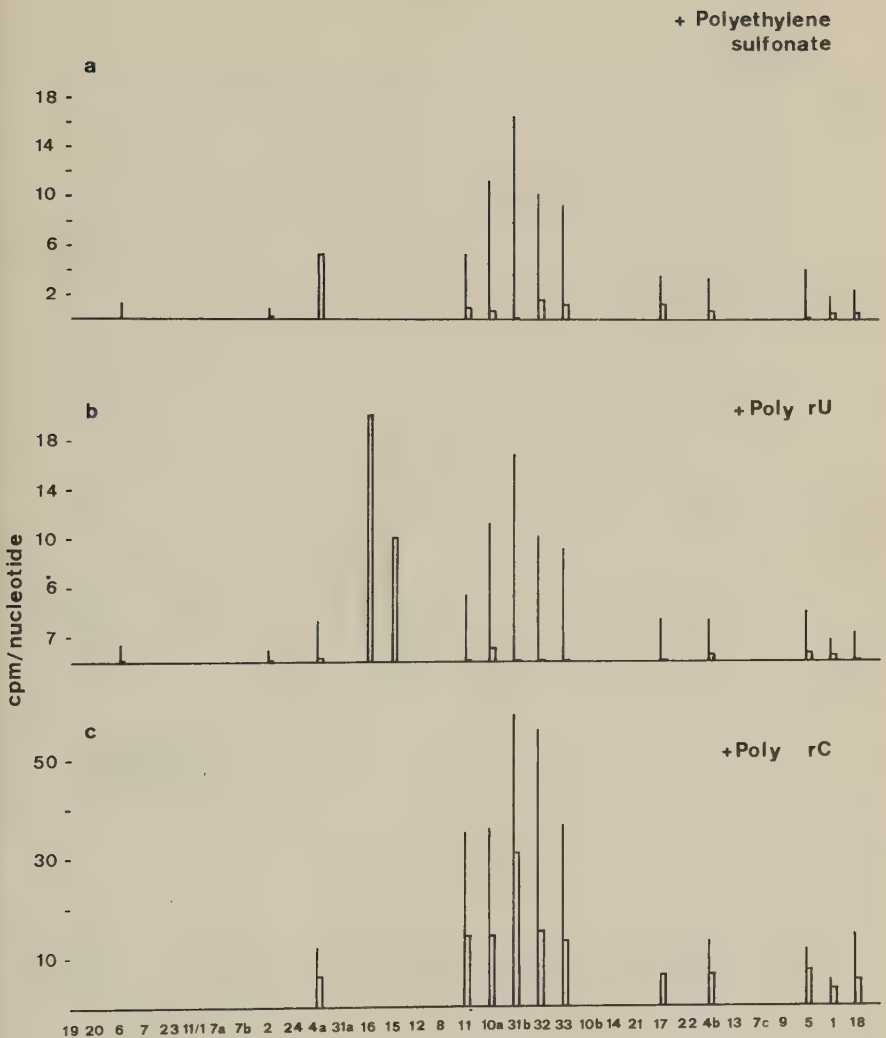
In the presence of poly(rU), the radioactivity retained on Millipore filter after nuclease digestion was reduced by 90%

Figure 3.15 Relative molar yields of ^{32}P-labeled T_1 oligonucleotides, obtained from ^{32}P-labeled Qβ RNA fragments bound to replicase in the presence of unlabeled RNA fragments M-2a/b and S-3, the homopolymers poly(rU) and poly(rC), and polyethylene sulfonate, after digestion with RNase T_1, plotted against their position on Qβ RNA.

A mixture containing 80 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 1 mM EDTA, 0.2 mg/ml BSA, 2 mM DTT, Qβ replicase (R) and unlabeled RNA fragments or polyethylene sulfonate (PES) in 40 µl (a-c) or 25 µl (d-f) were incubated for 3 min at 37°C. Initiation of Qβ RNA synthesis was allowed to occur by the addition of a solution containing in 10 µl (a-c) or 5 µl (d-f) ^{32}P-labeled Qβ RNA (Q) (specific activity: 2.5 10^6 cpm/µg), host factor I (HF) and GTP. After 20 sec of incubation, the reaction mixtures were diluted 5 fold into a solution containing 80 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 1 mM EDTA, 0.4 mM GTP, a five fold excess of unlabeled Qβ RNA over ^{32}P-labeled Qβ RNA and 1 unit of RNase T_1 per µg of RNA. After 10 min incubation at 25°C, the bound RNA fragments were isolated and completely digested by RNase T_1. The resulting oligonucleotides were fractionated and quantitated as described in the legend to Fig. 3.14. The relative molar yields are expressed in cpm per nucleotide for each oligonucleotide. Closed bars: without competitor present. Open bars: Binding in the presence of competitor.

a. 2.2 µg (R), 120 µg/ml PES. 5.2 µg (Q), 5 units (HF), 4.8 mM GTP
b. " , 1.9 µg poly(rU). " , " , "
c. " , 1.9 µg poly(rC). " , " , "
d. 0.45 µg(R), 0.4 µg M-2a/b. 0.9 µg (Q), 1 unit (HF) , 4.0 mM GTP
e. " , 0.4 µg S-3 . " , " , "
f. " , 0.4 µg rRNA/M. " , " , "

rRNA/M = E. coli ribosomal RNA fragments of the average size of M-2a/b.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18
14
10
6
2
b
+ Poly rU
18
14
10
6
7
cpm/nucleotide
c
+ Poly rC
50
30
10
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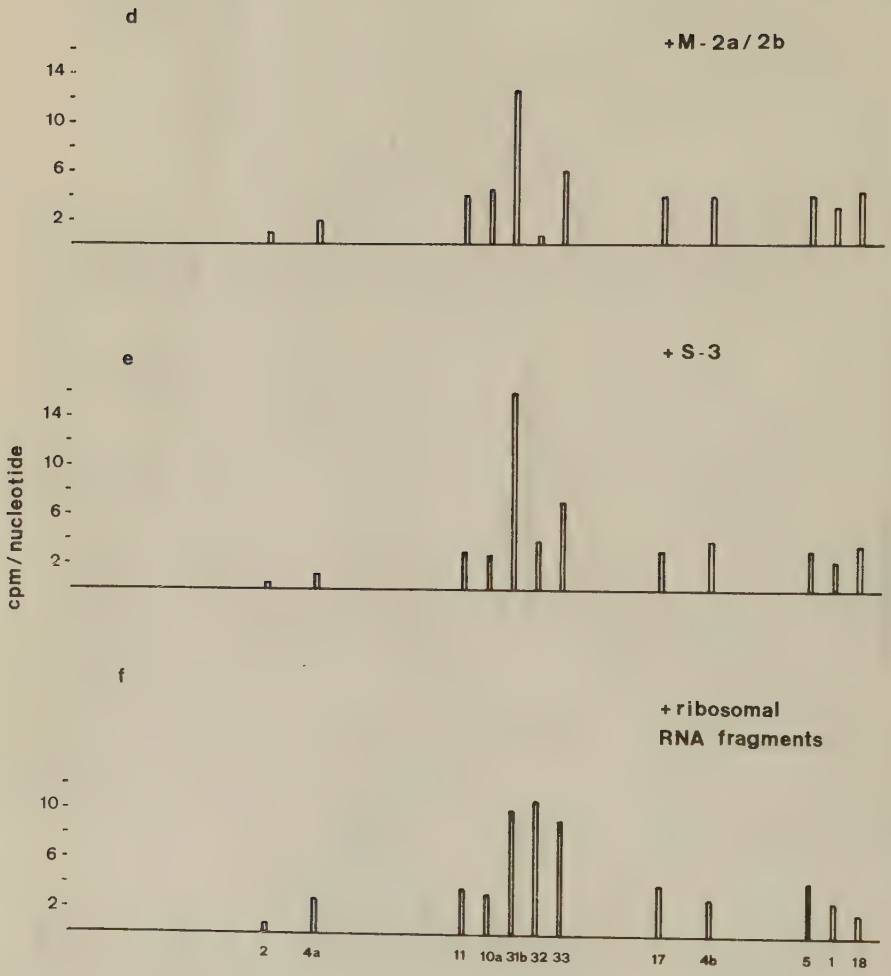
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+M-2a/2b
14
10
6
2
e
+S-3
cpm/nucleotide
14
10
6
2
f
+ribosomal
RNA fragments
10
6
2
2
4a
11
10a
31b
32
33
17
4b
5
1
18

compared to control. As can be seen in Fig. 3.15.b, poly(rU) appears to compete out all the interactions of replicase on the RNA. In the presence of poly(rU) additional oligonucleotides were bound to Qβ replicase (data not shown). They were found to have a high content of AMP residues and two of them had a partial nucleotide sequence consistent with T_1 oligonucleotides T15 and T16, respectively (see Table 3.5). The binding of these oligonucleotides to replicase may be mediated by poly(rU).

Poly(rC) diminished the radioactivity retained on Millipore filter after nuclease digestion by about 50% compared to control. The oligonucleotide pattern of the bound ^{32}P RNA remained unchanged (Fig. 3.15.c). Performing the digestion in the absence of excess unlabeled Qβ RNA, 80% of the radioactivity retained on the filter after nuclease digestion was competed out, however the oligonucleotide pattern remained essentially the same as in Fig. 3.15.c (data not shown).

When polyethylene sulfonate was present, the radioactivity retained on the filter after nuclease digestion was reduced by 80% compared to control. The residual 20% were due almost exclusively to S-site oligonucleotides (Fig.3.15.a). Polyethylene sulfonate appears to interfere with the enzyme in such a way that the interaction at the M-site and the 3'-end of Qβ RNA is inhibited, while the binding occurs almost exclusively at the S-site. By adding polyethylene sulfonate after the initiation-complex had been allowed to form, a binding pattern was obtained, consisting of only T4a (S-site), T17 and the 3'-terminal oligonucleotide T18. No quantitative analysis was done.

3.8 Replicase binding to RNase T_1 digested ^{32}P-labeled Qβ RNA

In the binding studies described so far, the interaction of replicase with intact Qβ RNA has been investigated. On the question of which structural features of the RNA, primary or higher order structure might be relevant for the interaction of the enzyme with the RNA, we investigated the binding of replicase to RNase T_1 digested Qβ RNA.

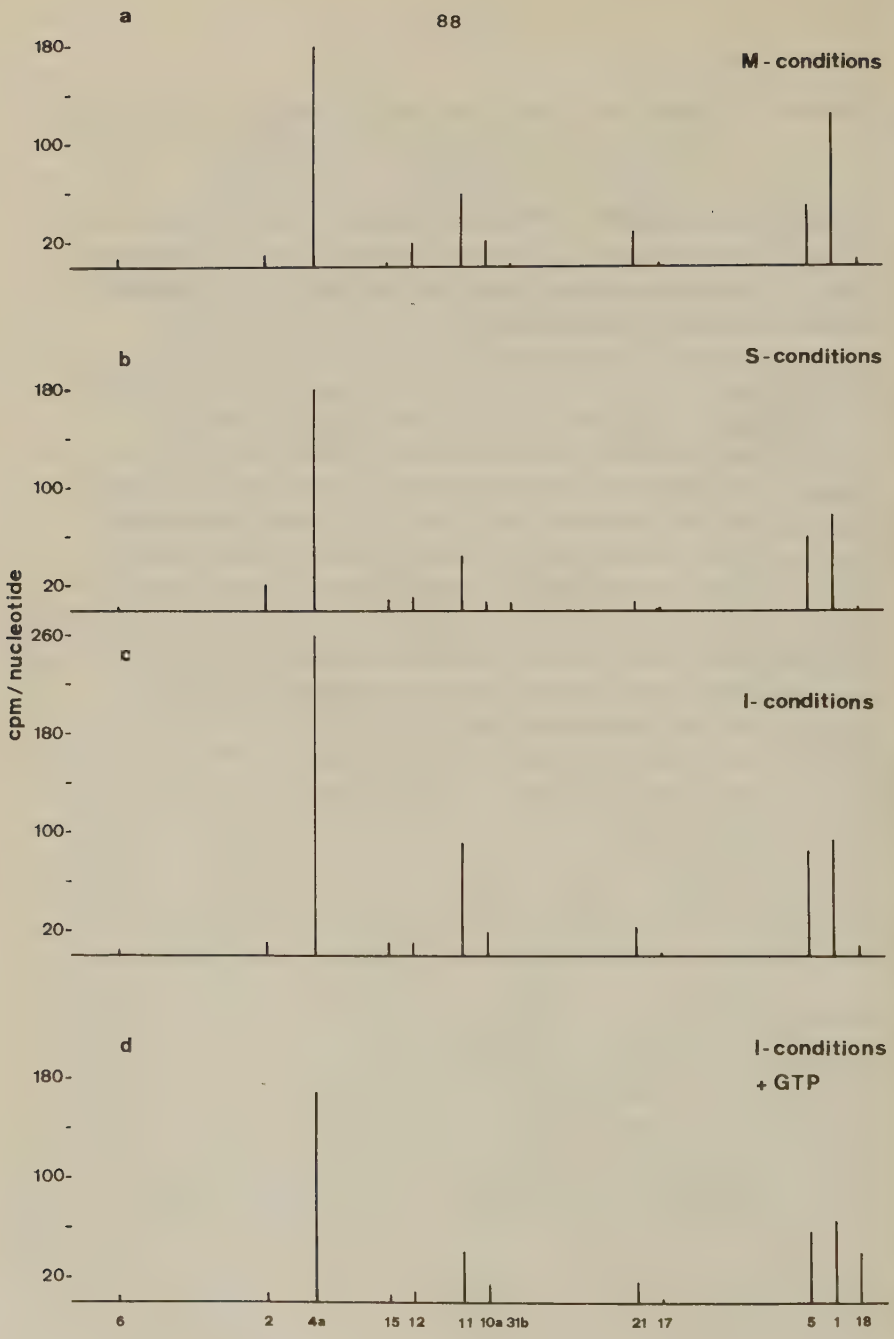
^{32}P Qβ RNA was digested to completion by RNase T_1 and the digest was incubated with Qβ replicase under various ionic conditions. The resulting complexes were filtered through Millipore filters and the retained T_1 oligonucleotides (0.23-0.4% of the input radioactivity under M-, S-, and I-conditions, 2.3-4% in the presence of Mn^{2+}-ions, 0.025-0.03% and 0.28%, respectively in the absence of replicase) were fractionated by 2 dimensional polyacrylamide gel electrophoresis and quantitated. The relative molar yields of the ^{32}P-labeled T_1 oligonucleotides were plotted against their position on Qβ RNA (Fig. 3.16). Under all ionic conditions used, replicase bound T_1 oligonucleotides (T4a, T11, T10a) derived from the internal binding sites on Qβ RNA (M-site and S-site) as well as T_1 oligonucleotides located in the 3'-terminal region (T5, and T1), but not T18, the terminal oligonucleotide. Under M-conditions, for instance, 1.4 pmoles T4a, 0.4 pmoles T11, 0.15 pmoles T10a, 0.6 pmoles T5 and 0.8 pmoles T1 were bound per 40 pmoles of replicase.

The qualitative binding pattern appears to be less sensitive to the ionic strength as compared to intact Qβ RNA.

In presence of host factor I, T17 was bound as well, which is in agreement with the findings of Senear and Steitz (1976). These authors demonstrated that host factor binds specifically to the oligonucleotides T17 and T1, when Qβ RNA or Qβ RNA which had been digested to completion by RNase T_1 was incubated with

Figure 3.16 Relative molar yields of ^{32}P-labeled T_1 oligonucleotides, obtained from binding Qβ replicase to RNase T_1 digested ^{32}P-labeled Qβ RNA.

10 µg ^{32}P-labeled RNase T_1 digested Qβ RNA (prepared as described in Methods, section 2.2.3; specific activity: 1.7 10^6 cpm/µg) and 9 µg Qβ replicase were incubated for 5 min at 37°C under M-conditions (a), S-conditions (b), I-conditions (c), I-conditions with 10 units of host factor I (e), I-conditions with 0.4 mM GTP (d), I-conditions with 0.4 mM GTP and 10 units of host factor I (f), Mn^{2+}-conditions (g), Mn^{2+}-conditions with 0.4 mM GTP (h). The reaction mixtures were filtered through Millipore filters, which had been preincubated in the corresponding buffers containing 0.5 mg/ml yeast carrier RNA and the filters were washed 3 times with 1 ml of the corresponding buffer. The bound T_1 oligonucleotides were extracted, fractionated by 2 dimensional polyacrylamide gel electrophoresis and quantitated as described in Methods, section 2.2.1. The relative molar yields of the bound T_1 oligonucleotides are expressed in cpm per nucleotide.
For M-, S-, I- and Mn-conditions, see Methods, section 2.2.1.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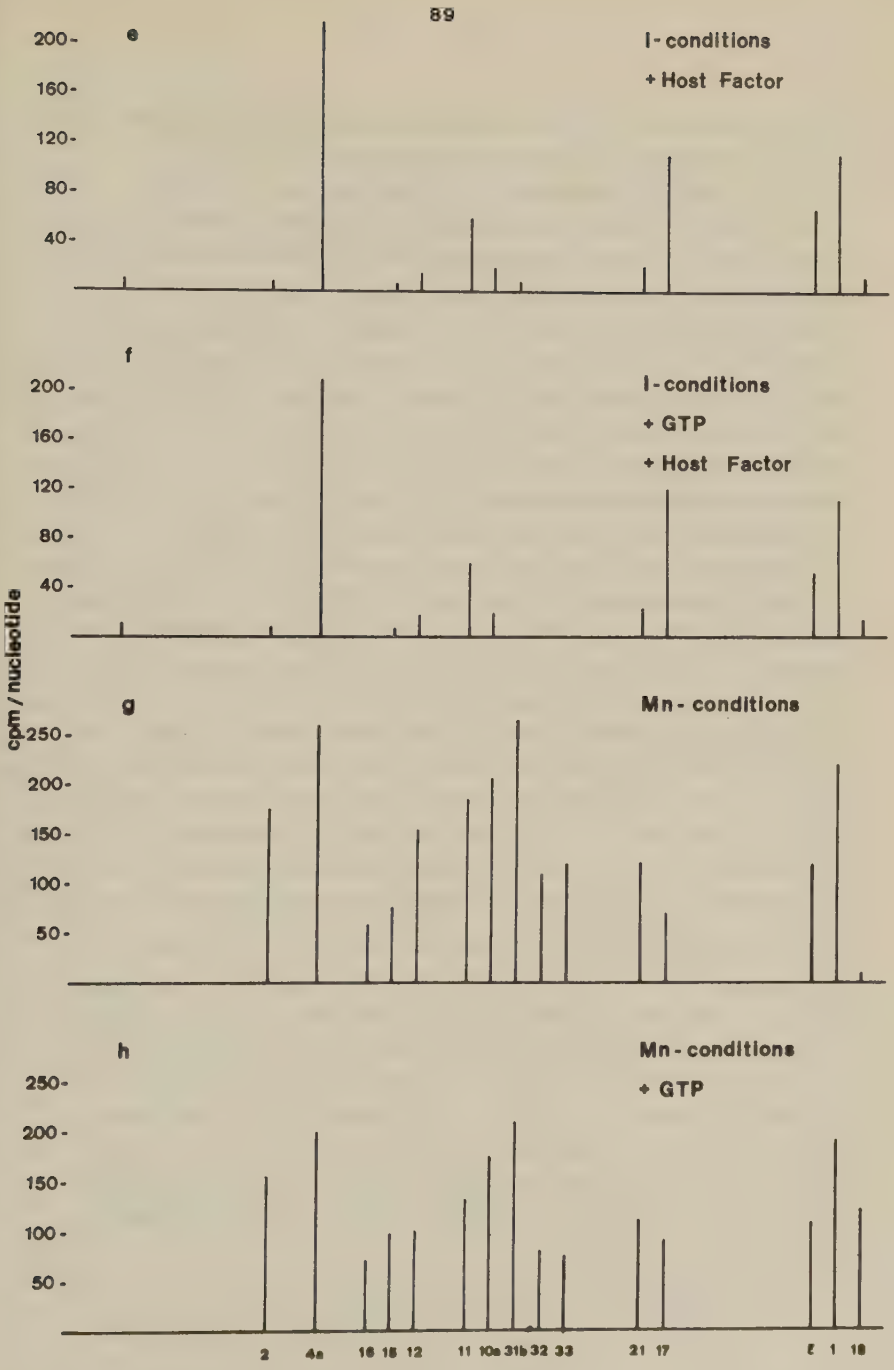
180-
100-
20-
b
S - conditions
180-
100-
20-
cpm / nucleotide
260-
c
I - conditions
180-
100-
20-
d
I - conditions
+ GTP
180-
100-
20-
6
2
4a
15 12
11 10a 31b
21 17
5 1 18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I - conditions
+ Host Factor
f
I - conditions
+ GTP
+ Host Factor
cpm / nucleotide
g
Mn - conditions
h
Mn - conditions
+ GTP
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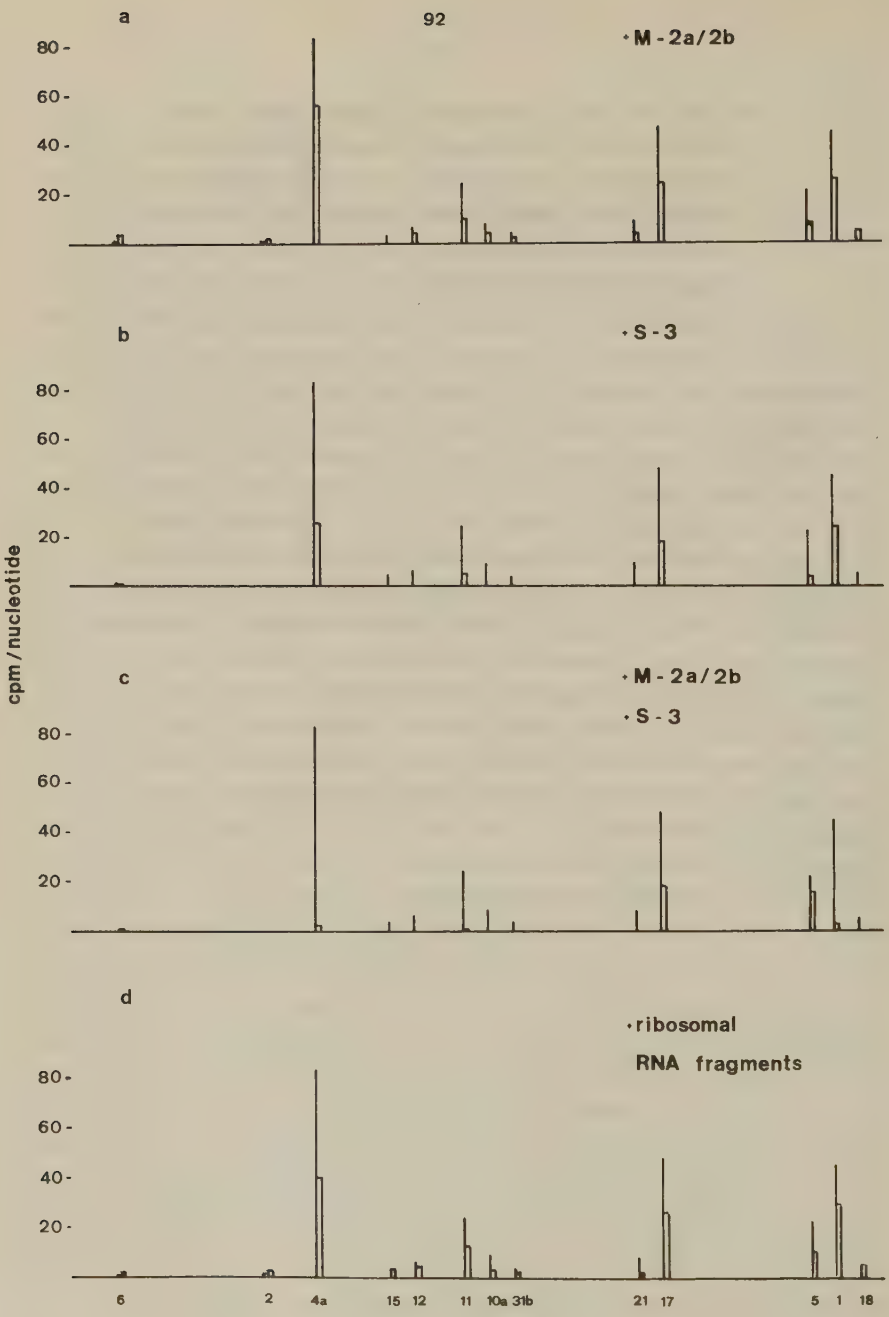
80
40
250
150
100
50
2
4a
16
15
12
11
10a
31b
32
33
21
17
E
1
18

host factor I and the resulting binding-complexes analysed by a similar approach as described above.
T31b, T32 and T33 were absent, in contrast to the binding pattern obtained with intact Qβ RNA (Fig. 3.13 and Fig. 3.14), suggesting that these oligonucleotides are not involved in a direct interaction with the enzyme, but rather are part of a larger sequence which might be involved in the binding. Alternatively the interaction with these RNA fragments could be due to the secondary or tertiary structure of intact Qβ RNA.
When Mn^{2+}-ions were present, the affinity of the enzyme to the oligonucleotides was increased 2-10 fold in general, compared to conditions where Mg^{2+} or salt was present, however the binding specificity appears to be reduced. Oligonucleotides showing insignificant binding under conditions where Mn^{2+} was absent had a relatively high affinity to the enzyme in the presence of Mn^{2+} (T2, T15, T12, T21). Furthermore addition of GTP to the reaction mixture increased the binding of the 3'-terminal oligonucleotide T18 to the enzyme about 12 fold (see Fig. 3.16.g and h). The fact that oligonucleotides with a chain length as short as 17 nucleotides are bound specifically suggests that replicase recognizes specific nucleotide sequences. This of course does not exclude the recognition of defined secondary and or tertiary structure as well.

Next it was of interest to test how the binding of these T_1 oligonucleotides was affected by the RNA fragments M-2a/b and S-3 or by the homopolymers poly(rU) and poly(rC) or polyethylene sulfonate. For this purpose replicase was preincubated with the various RNA fragments and polyethylene sulfonate and subsequently RNase T_1 digested ^{32}P-labeled Qβ RNA was added in the presence of host factor I and GTP. Incubation was for 90 sec (to minimize the exchange between the fragments) and the binding mixture was filtered through Millipore filters.

Figure 3.17 Relative molar yields of ^{32}P-labeled T_1 oligonucleotides from RNase T_1 digested ^{32}P-labeled Qβ RNA bound to Qβ replicase in the presence of excess unlabeled fragment M-2a/b, S-3, E. coli ribosomal RNA fragments, poly(rU), poly(rC) and polyethylene sulfonate.

3 μg Qβ replicase and 0.4 μg M-2a/b (a), 0.4 μg S-3 (b), 0.4 μg M-2a/b and 0.4 μg S-3 (c), 0.4 μg E. coli ribosomal RNA fragments of the average size of S-3 (d), 125 μg/ml polyethylene sulfonate (e), 1.0 μg poly(rU), average size: 130 nucleotides (f), 1.0 μg poly(rC), average size: 130 nucleotides (g) were incubated for 3 min at 37 °C in a 40 μl reaction mixture in I-buffer containing 0.4 mM GTP. 10 μl of a solution (preincubated for 3 min at 37°C) containing 4 μg ^{32}P-labeled RNase T_1 digested Qβ RNA (prepared as described in Methods; specific activity: 1.7 10^6 cpm/μg), 4 units of host factor I, 0.4 mM GTP in I-buffer was added and the binding was allowed to occur for 90 sec. The reaction mixtures were filtered and further processed as described in the legend to Fig. 3.16. The RNA fragments M-2a/b, S-3, E. coli ribosomal RNA fragments and the homopolymers poly(rU), poly(rC) were prepared as described in Methods.
Closed bars: Binding without competitor present; open bars: Binding in the presence of competitor.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40-
20-
b
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c
·M-2a/2b
·S-3
80-
60-
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d
·ribosomal
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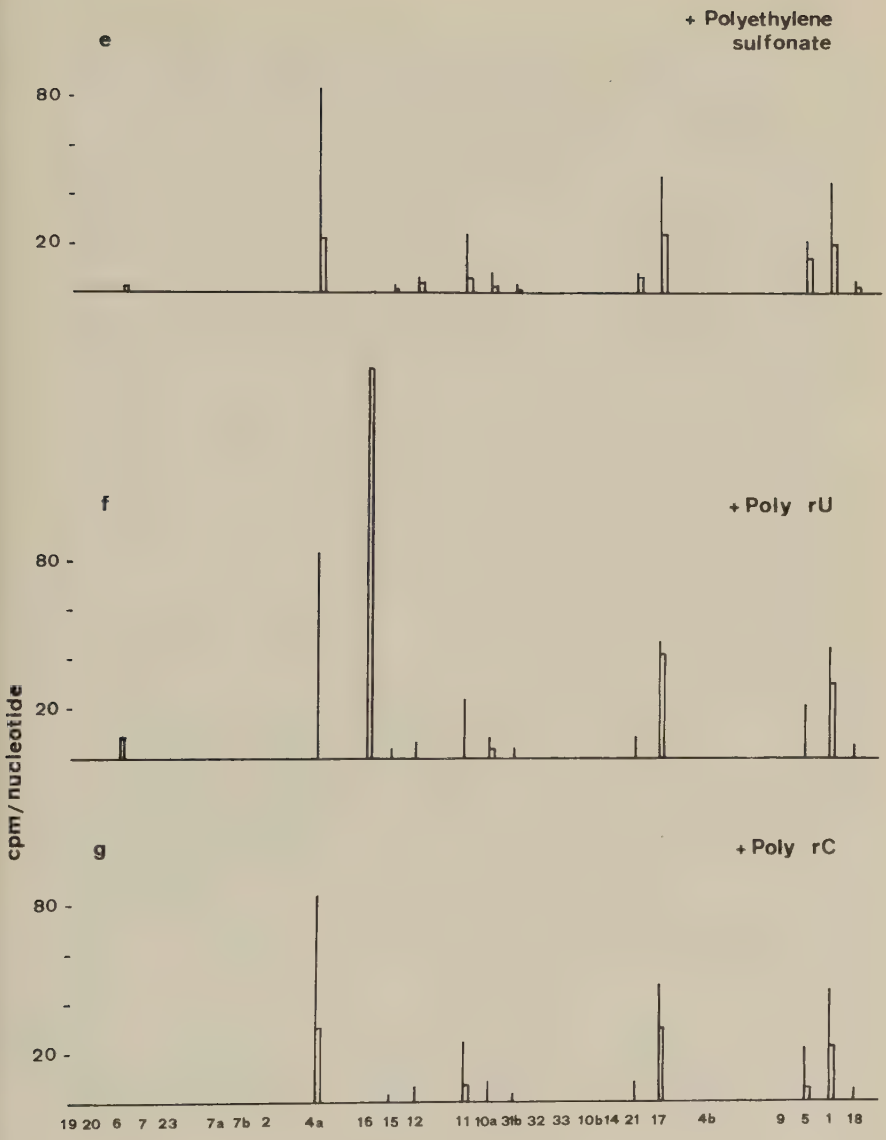
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+ Polyethylene sulfonate
80
20
f
+ Poly rU
80
20
cpm / nucleotide
g
+ Poly rC
80
20
19 20 6 7 23 7a 7b 2 4a 16 15 12 11 10a 3tb 32 33 10b 14 21 17 4b 9 5 1 18

The bound fragments were isolated as usual and fractionated by 2 dimensional polyacrylamide gel electrophoresis. The relative molar yields of the various oligonucleotides were plotted against their position on Qβ RNA (Fig. 3.17). In the presence of the RNA fragments M-2a/b or S-3 at a molar excess over RNase T_1 digested Qβ RNA of 2.8 and 4.5, respectively, the binding of all T_1 oligonucleotides was affected to various extents (Fig. 3.17.a,b). However when E. coli ribosomal RNA fragments of the average size of S-3 were added at a 4.5 fold molar excess over RNase T_1 digested Qβ RNA, the binding pattern looked similar to the one obtained in the presence of the specific fragments M-2a/b or S-3 (Fig. 3.17.d).

In the presence of polyethylene sulfonate no specific competition could be seen (Fig. 3.17.e), but rather a general decrease in the binding of the T_1 oligonucleotides. Poly(rC) and poly(rU) which had been sized to about 130 nucleotides, were used at an 8 fold molar excess over RNase T_1 digested Qβ RNA. Poly(rC) reduced the binding of the major T_1 oligonucleotides by about 50% (Fig. 3.17.g), whereas in the presence of poly(rU) (Fig. 3.17.f), T4a (S-site), T11 (M-site) and T5 (3'-terminal region) were undetectable. T17 and T1 were not significantly competed. However in the presence of poly(rU) additional oligonucleotides having a high content in AMP residues were bound to replicase (not shown). One of these had a partial sequence consistent with T_1 oligonucleotide T16 (Table 3.5). Others having stretches of 3-4 AMP residues were not further analysed. The binding of these oligonucleotides to replicase may be mediated by poly(rU).

4. DISCUSSION

1) Replicase binds at two internal sites on Qβ RNA

The interaction between Qβ replicase and Qβ RNA was studied using the binding assay described by Weber et al, (1972). In such an assay replicase is bound to ^{32}P-labeled RNA and the enzyme-RNA complexes are digested by RNase T_1 in the presence of excess unlabeled RNA to diminish any unspecific binding of digested labeled RNA to replicase. RNA segments recovered under these conditions are not necessarily protected against nuclease along their entire length but may be partial degradation products remaining selectively attached to the enzyme.

Using different ionic conditions (S-conditions and M-conditions) two different oligonucleotide patterns were found by analysing the bound RNA fragments on a polyacrylamide gel (see Fig.3.2): The pattern obtained under S-conditions was identical to the one described by Weber et al, (1972). It represented a set of RNA fragments derived from a sequence à 100 nucleotides in length (S-site) located at the beginning of the coat cistron. The binding does not require Mg^{2+}-ions, but monovalent ions and incubation at 37°C (Weber et al., 1974). The interaction at this site is thought to be involved in the replicase repressor function (Kolakofsky & Weissmann, 1971b).
The pattern obtained under M-conditions was more complex. The size of the retained fragments ranged from 21 to 173 nucleotides in length. Among the major bands present there was no fragment derived from the S-site. The M-fragments are derived from a circumscribed segment of Qβ RNA located between 2100-2600 nucleotides from the 5'-end of Qβ RNA. According to both our mapping experiment and the T_1 oligonucleotide map of Qβ RNA (Billeter, M.A., unpublished results), the M-site is located within the replicase cistron, not far from its starting point.

The specificity of the interaction at the S- and M-site was established in rebinding experiments. The largest major oligonucleotides of the binding sites (RNA fragments S-3 and M-2a/b, Fig. 3.2) were specifically rebound to replicase in the presence of a vast excess of alkali degraded Qβ RNA fragments of the same size as the specific fragments. The binding efficiences of the RNA fragments from the binding sites were comparable to the binding efficiences obtained by using intact Qβ RNA, suggesting that the information necessary for the interaction was retained in these fragments.

The binding of RNA fragments M-2a/b and S-3 to replicase was competitive. The competition was specific inasmuch as ribosomal RNA fragments of the same average size did not significantly compete the binding of the RNA fragments M-2a/b or S-3. Furthermore it was found, that the RNA fragments M-2a/b and S-3 did not bind additively to the enzyme. These results suggest that these RNA fragments interact at either the same binding site on the enzyme or if there are two or more binding sites for the 2 fragments, that their binding is mutually exclusive.

The next important question was whether the strong and specific interactions of replicase at the 2 internal sites on Qβ RNA had any functional role in Qβ RNA synthesis. Previous results had suggested that 3'-terminal fragments of Qβ RNA function as replicase templates provided they are longer than about 50% of the total RNA length (Schwyzer et al., 1972). Since the M-site is located around the middle of Qβ RNA we suspected that the M-site might be required for template recognition whereas the S-site, located closer to the 5'-terminus would not be essential. This conclusion was furthermore strengthened by the finding of a deletion mutant of Qβ RNA, obtained after prolonged replication in vitro which lacked the S-site as judged by the absence of T_1 oligonucleotides T7 to T8 (see Fig. 3.12) from the fingerprint (Sabo et al.,1977).

This RNA molecule was very efficiently replicated despite the deletion.

We studied the effect of the RNA fragments M-2a/b and S-3, derived from the internal binding sites M and S, respectively on the initiation of Qβ RNA replication. It was found that both RNA fragments, but not fragments of E.coli ribosomal RNA, inhibited initiation of Qβ RNA synthesis. Alkali degraded Qβ RNA preparations of the same average size as the fragments M-2a/b and S-3, respectively,were 2 to 5 times less effective. The RNA fragments did not affect the reaction after chain initiation had occured. These findings suggest that the RNA fragments from the binding sites specifically inhibit the formation of a productive initiation complex by preventing Qβ RNA from binding correctly to replicase.

Why do both S- and M-site RNA fragments inhibit initiation of Qβ RNA synthesis, although the interaction at S-site seems not to be essential? Unfortunately, one important piece of information is still lacking, namely the number of binding sites for internal RNA sequences on the enzyme molecule. We therefore attempt to discuss the significance of all these different results in terms of several models which differ in regard to the number of binding sites and their specificities for M- and S-site (Fig. 4.1). Models(a)(b)(c) postulate two binding sites on replicase: (a),one specific for M-site and one specific for S-site. The binding at one site is not affected by the binding at the other site. (b),both sites bind S-site and M-site equally well. The binding at one site is not affected by the binding at the other site. (c),one specific for M-site and one specific for S-site. The binding at the two sites is mutually exclusive (allosteric interaction). In model (d) there is only one binding site on the enzyme which binds either S-site or M-site.

As already mentioned, the binding of the 2 RNA fragments

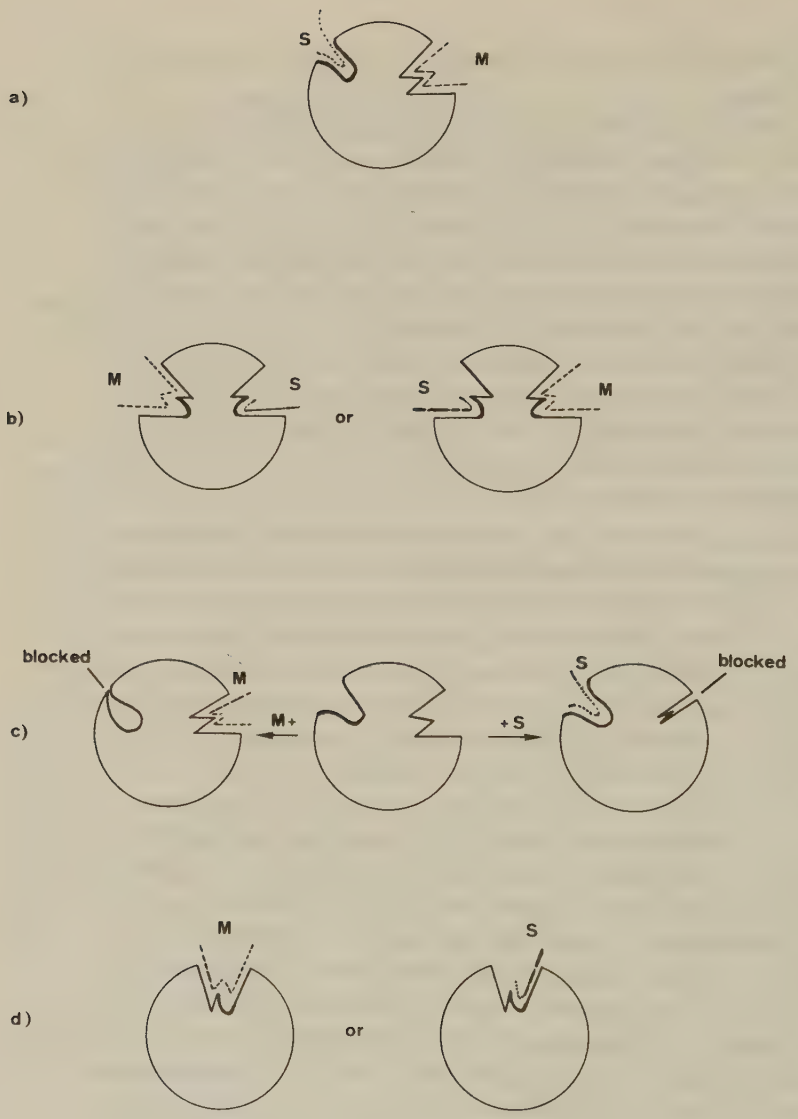

Figure 4.1 Models for the interaction of replicase with M-site and S-site.

derived from M-site and S-site, was found to be competitive. This finding excludes model (a). At present, we cannot discriminate between models (b), (c) and (d), as it has been experimentally impossible to determine whether one enzyme molecule binds to both RNA sites simultaneously (model (b)) or at either site alone (model (c) and (d)).

Electron microscopic visualization of binding complexes, which were prepared under ionic conditions where an interaction at both sites was demonstrated biochemically (I-conditions), and cross-linked by formaldehyde, showed complexes with a single internal loop in the RNA with the replicase bound at its base (Vollenweider et al., 1977). The positions of the binding sites identified by electron microscopy were found compatible with the locations of the M- and S-site, as determined by biochemical methods. These data would be in agreement with model (a) and model (b). However as the authors point out, it is not excluded that replicase specifically bound to one of the binding sites could be crosslinked by formaldehyde to a spatially (but not linearly) adjacent RNA region not necessarily identical with the second biochemically identified site.

Models (b), (c) and (d) are all compatible with the finding that both RNA fragments (M 2a/b and S-3) inhibit initiation of Qβ RNA synthesis: Prebinding one of the two RNA fragments abolishes the binding capacity of the enzyme to the other site concomitantly, such that Qβ RNA can no longer bind correctly to the enzyme.

2) Nucleotide sequence and structure of replicase binding sites

In order to get some insight regarding which structural features of the RNA might be involved in the recognition process between Qβ replicase and Qβ RNA, the complete nucleotide

sequence of the major RNA fragment M-2a/b (M-site) was determined.

By comparing the nucleotide sequences and the secondary structures of the RNA fragments from the M- and S-sites of Qβ RNA, no significant sequence homologies or secondary structure similarities were found.

The low degree of secondary structure (the self-complementary regions were deduced according to Tinoco et al. 1971) in fragment M-2a/b (13% base-pairing) might be a significant characteristic, however other RNA fragments derived from the M site and S-site show a more extensive secondary structure (M-5: 50% base-pairing, M-11: 38% base-pairing, S-3: 44% base-pairing). Furthermore these RNA regions, as part of the complete RNA could be involved in secondary structure with other distant regions.

Some complementary sequences between M-site and S-site RNA fragments could be detected and are shown in Fig. 4.2.

M-2/S-3 (ΔG=-22.4kcal)

$$\begin{array}{l} U_{GAAUGUG}{}^{C}{}_{UUUAA\ CGAAC}{}^{G} \\ U^{CUUGCAC\ AAAUU}{}_{U}{}^{GCUUG}{}_{A} \end{array}$$

M-2/M-5 (ΔG=-14.4kcal)

$$\begin{array}{l} {}^{AG}{}_{AGGCUGAA}{}^{UG} \\ {}_{GA}{}^{UCCGACUU}{}_{CA} \end{array}$$

S-3/M-5 (ΔG=-10.8kcal)

$$\begin{array}{l} AAA_{CGGU}{}^{A}{}_{CUAGU}{}^{UU} \\ AAA^{GCCG}{}_{A}{}^{GAUCA}{}_{UG} \end{array}$$

M-5/M-11 (ΔG=-9.0kcal)

$$\begin{array}{l} {}_{G}{}_{ACUU}{}^{U}{}_{AGGA}{}^{UA} \\ {}^{UGAA}{}_{CU}{}^{UCCU}{}_{GC} \end{array}$$

Figure 4.2 Complementary regions between S- and M-site RNA fragments, derived according to Tinoco et al.(1971)

However, except for the duplex S-3/M-5, one of the two strands

is already involved in a secondary structure stem of similar stability by itself. Furthermore M-site RNA fragments could be isolated without S-site fragment and vice versa, suggesting that these complementary regions between the S-site and M-site RNA fragments are unlikely to lead to secondary structure.

Qβ replicase was shown to bind to internal regions of 6s nano-variant RNA (Schaffner, 1977), MS2 RNA (Vollenweider, H.J., Weber,H., Fiers,W. & Weissmann, C., unpublished experiments) and MDV-1 (Mills et al., 1977). However there are no significant sequence and secondary structure homologies between the binding sites found in these RNA molecules and Qβ RNA. Thus no general conclusions as to the molecular mechanism of the enzyme recognition process can be drawn so far by comparing sequence and secondary structure of known binding sites.

On the other hand, the results of a different experimental approach suggest, that as far as single sites are concerned, replicase does not recognize secondary and/or tertiary structural elements but rather specific nucleotide sequences. This was demonstrated by the finding that after complete digestion of Qβ RNA with ribonuclease T_1, replicase selectively bound to large T_1 oligonucleotides derived from the internal binding sites (S-site and M-site) as well as the large T_1 oligonucleotides derived from the 3'-terminal region. These oligonucleotides are the same as the ones obtained where intact Qβ RNA was bound to Qβ replicase and the retained fragments fingerprinted (Fig.3.13).
The major T_1 oligonucleotides bound to replicase under M-, I- and S-conditions were the following:

T1 AAUAAAUUAUCACAAUUACUCUUACG (3'-terminal region)
T4a AUCUUUUUAUUAACCCAACG (S-site)
T5 UCCUAAUCAAUCCUUUCG (end of replicase cistron)
T11 AUUUCAAUUUCUCACUG (M-site)

The oligonucleotides bound are variable in size (other oligonucleotides of the same size were not bound) suggesting that the selective binding is not due to a particular length distribution, but rather a sequence selection.
Other T_1 oligonucleotides, which were bound to replicase with a 10 to 20 fold lower affinity relative to T4a (the oligonucleotide bound with the highest affinity) are not shown (however see Fig. 3.16). The relative binding efficiencies of the various oligonucleotides in decreasing order are as follows: T4a > T1 > T5 > T11 > T21 > T10a > T12 > T2 > T18

Two sequence homologies between the various oligonucleotides were detected:
a) Sequence homology between T_1 and T10a:

```
T1:          AAUUAAAUUAUCACAAUUACUCUUACG
                ////////
T10a:         AUAAAUUAUCUUAAAG
```

10 nucleotides out of 16 from the 5'-end of T10a (derived from the fragment M-5, Fig.3.2) are identical to the 5'-end of T1 (derived from the 3'-terminal region of Qβ RNA, Fig. 3.12). T10a, however was not efficiently bound by replicase out of the complete RNase T_1 digest of Qβ RNA, which suggests that the common 10 nucleotides are not directly involved in a stable interaction with Qβ replicase.

b) Sequence homology between T10a and T12:

```
T10a          AUAAAUUAUCUUAAAG
                 ||||||||||
T12          AUUCAUUUAUCUUAAG
```

9 nucleotides out of 16 from the 3'-end of T10a are identical to the 3'-terminal region of T12. It might be noted that these two T_1 oligonucleotides are not bound efficiently to replicase.
Whereas no further sequence homology between the major bound

T1 oligonucleotides was found, the following striking sequence arrangement might be mentioned:

a) UAPyPyPyPyUA	present in T1
b) AAPyPyPyPyUPyA	present in T11
c) $\overset{U}{A}$APyPyPyPyUPyPyPu	present in T4a and T5

Each of these oligonucleotides has an A residue (0) and an U residue (5), which are separated by four pyrimidines. At position (-1) only U or A are found. Not more than 7 pyrimidines separating A (0) and the next purine and not less than 5 pyrimidines between any two A residues are found. By comparing the nucleotide sequence of all the known large T_1 oligonucleotides of Qβ RNA (a total of 29, as determined by Billeter, M.A., unpublished results) with the sequences a) to c), it was found that no additional T_1 oligonucleotide contained a sequence of this sort, except T2 (see Table 3.5.) which contains the sequence -AAAPyPyPyPyUPyA- and which bound to replicase about 20 times less efficiently than T4a. The fact that only the four T_1 oligonucleotides (T4a, T1, T5 and T11), which were selectively bound to replicase (except T2) contain this particular sequence out of 29 T_1 oligonucleotides, raises the question as to whether this structure might be a "recognition site" for Qβ replicase on Qβ RNA. If this were so, in order to explain why T2 was not significantly bound to replicase, one has to assume that the flanking nucleotides on either side of this structure are important to allow a further discrimination: a "wrong" nucleotide or blocks of nucleotides at certain positions might destabilize the binding complex due to sterical and/or electrostatic restrictions.

Assuming one binding site on replicase, one is tempted to speculate that the specificity in the interaction between replicase and an oligonucleotide is not determined by a unique

nucleotide sequence, which would have to be common to all selectively bound oligonucleotides, but a nucleotide sequence exhibiting common features, such as a stretch rich in pyrimidines or alternatively a particular arrangement of amino and/or carbonyl groups, which fulfill the sterical and/or electrostatic requirements of the enzyme binding site. In this connection it might be noted that the synthetic homopolymers, poly(rU) and poly(rC) of similar size as the internal binding site RNA fragments M-2a/b and S-3, compete with the binding of these RNA fragments to Qβ replicase, showing that the homopolymers have an affinity for the M- and S-site fragment binding site(s). According to this model replicase might have a binding affinity to a large variety of oligonucleotides. More information might be gained by establishing a larger catalog of replicase bound oligonucleotides from different RNA molecules. It seems appealing to bind replicase to T_1 oligonucleotides derived from different RNA molecules like Qβ RNA minus strand, 6s nanovariant RNA (plus and minus strand),MDV-1(plus and minus strand),where replicase binding studies have been carried out using intact RNA as substrate (Mills et al., 1977; Schaffner, 1977; Müller, W., Weber, H. & Weissmann, C., unpublished results). Binding studies with degraded oligonucleotides or chemically synthesized specific oligonucleotides might help define the sequences directly involved in the interaction.

An alternative explanation for not finding sequence homologies between the bound T_1 oligonucleotides would be that they are interacting at different binding sites on Qβ replicase. As this question cannot be answered at present, additional information might be gained from binding competition experiments between the various T_1 oligonucleotides. Furthermore crosslinking of the various oligonucleotides to the protein moiety and identification of the crosslinked amino acids or polypeptides could give information on the amino acids of the binding sites

involved in the interaction and determine whether the binding sites are located on different or the same subunit of Qβ replicase. In this connection it may be noted that purified S_1, the α-subunit of replicase, selectively bound the T_1 oligonucleotides T1 (3'-terminal region) and T4a(S-site) out of a complete RNase T_1 digest of Qβ RNA (Sénear & Steitz, 1976; Goelz & Steitz, 1977), suggesting that the interaction of replicase at these oligonucleotides is due to a direct interaction of S_1 protein with these sequences.

It remains to be demonstrated whether the selectively bound T_1 oligonucleotides show a specific inhibitory effect on Qβ RNA synthesis, as it had been found for the binding site RNA fragments M-2a/b and S-3.

3) Association of Qβ replicase and Qβ RNA under initiation conditions: Binding of the 3'-end

For the initiation of Qβ RNA synthesis i.e. the formation of the first phosphodiester bond, in addition to Qβ replicase the bacterial protein, host factor I as well as GTP are required (initiation conditions). The function of host factor I is not required for synthesis directed by the Qβ complementary RNA or other templates for the enzyme.

The nature of the interactions between replicase and Qβ RNA under initiation conditions was studied by analysing the whole collection of replicase-bound RNA fragments by T_1 fingerprinting. This allowed the identification of those RNA segments containing easily identifiable large T_1 oligonucleotides in particular S- and M-site RNA fragments and the 3'-terminal region.

These experiments showed that under initiation conditions the interaction between replicase and Qβ RNA involved 3 main regions on the RNA: The 2 internal sites (S-site and M-site),

described above and the 3'-terminal region including the 3'-terminus. Including ATP in the reaction mixture gave a similar result.

Polyethylene sulfonate inhibits chain initiation but not chain elongation (Kondo & Weissmann, 1972);RNA binding was found to be far less sensitive to polyethylene sulfonate inhibition than chain initiation. We have studied the effect of polyethylene sulfonate on Qβ RNA binding to replicase, using our binding assay.

Preincubating Qβ replicase with polyethylene sulfonate and subsequently adding radioactively labeled Qβ RNA under conditions where initiation can occur,showed that the binding of both the M-site and the 3'-terminal region as well as the host factor stimulated binding regions near the 3'-end was inhibited. The interaction at the S-site (the only one still detectable) appeared to be enhanced.

On the other hand if polyethylene sulfonate was added after initiation of replication, RNA binding at the 3'-terminus as well as at the S-site was detected, while the interactions at the M-site were inhibited. These observations suggest that the binding at the M-site but not at the S-site is required for initiation. It furthermore explains the differential inhibitory effect of polyethylene sulfonate on Qβ RNA binding and initiation of Qβ RNA synthesis.

These experiments show that only when initiation of Qβ RNA synthesis is allowed to occur an interaction at the 3'-terminus is taking place. About 7% of the RNA present in the binding mixture had the 3'-end bound to the enzyme. Since under these reaction conditions only 10 to 15% of the RNA are capable of initiating RNA synthesis, these results indicate a direct correlation between complex formation at the 3'-terminus and initiation of Qβ RNA synthesis.

It cannot be established from these experiments whether

synthesis is required for the 3'-end binding or whether GTP binding in the presence of host factor would be sufficient. If synthesis were necessary, substituting GTP by GMPCPP, an analog of GTP which prevents the condensation reaction to occur, should result in an inhibition of the 3'-end binding to replicase. It seems rather likely that the interaction of the 3'-end with replicase is mediated via product binding.

Senear & Steitz (1976) and Goelz & Steitz (1977), have shown that host factor interacts specifically with Qβ RNA at the 3'-terminal region at the sequences corresponding to the T_1 oligonucleotides T17 and T1 (see Fig. 3.12). In addition ribosomal protein S_1, the α-subunit of Qβ replicase was found to bind to the 3'-terminal region and to the internal binding site (S-site) at the sequences corresponding to the T_1 oligonucleotides T1 and T4a, respectively. Our data showed that although S_1 is part of replicase only T4a (S-site) and T_1 oligonucleotides from the M-site showed a strong interaction with replicase, whereas the T_1 oligonucleotides from the 3'-terminal region including the 3'-terminus were only bound to the enzyme under initiation conditions.
Thus although subunit S_1 and host factor by themselves can recognize a 3'-terminal sequence on Qβ RNA, when the RNA is bound to the complete enzyme, this site is probably masked due to the RNA structure, since upon disrupting Qβ RNA by complete digestion with RNase T_1, the T_1 oligonucleotide T1 is selectively bound to replicase.

In this connection it might be mentioned that Qβ replicase lacking α-subunit was found to bind less tightly to Qβ RNA than the complete enzyme. Digestion of the resulting complexes with RNase T_1 did not yield specific RNA fragments as evidenced by the absence of defined bands on polyacrylamide gels. When α-subunit and the subunit defficient enzyme were simultaneously added to the binding reactions, RNA fragments indistinguishable from those found with the complete replicase were obtained

(Weber,H., Kamen,R. & Weissmann,C., unpublished experiments). These data suggest that ribosomal protein S_1 is required for tight binding of the replicase to the internal binding sites, M-site and S-site. Since the interaction of replicase at the S-site can be traced to a direct interaction of S_1 protein (see above), only the interaction of replicase to the M-site remains to be explained in terms of a specific recognition by one of the enzyme subunits. It is conceivable that when S_1 is part of replicase, S_1 can interact at the M-site or S_1 promotes an interaction at the M-site by a different subunit.

4) Model for template recognition

A model for template recognition has been proposed (Weissmann, 1974), which postulated that template recognition by replicase involves the tight binding of the RNA at an internal sequence (located around the middle of the genome) such that the 3'-end which by itself has a low affinity for replicase is positioned at the enzyme initiation site where chain initiation occurs (Fig. 4.3). This model accounts for the fact that other RNAs such as MS2, R17 or f2 RNA, which have relatively high affinities for replicase and similar 3'-termini do not allow any initiation of RNA synthesis

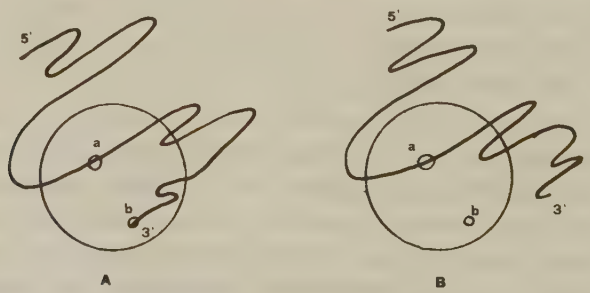

Figure 4.3 Model for template recognition by Qβ replicase

Two sites on the enzyme are required for template recognition (the site responsible for translational repression, S-site, is not shown).
(A): Productive interaction with Qβ RNA. Site (a) binds an internal region of the RNA tightly, placing the 3'-terminus in site (b), where it is weakly bound and chain initiation can occur.
(B): Non-productive interaction with heterologous RNA: Site (a) binds an internal region of the RNA, for which it has some affinity, however the 3'-terminus is located far from site (b) and initiation cannot take place (The figure is taken from Weissmann, 1974).

The results of the present work basically confirm the model of Fig. 4.3.

Based on the previously described models (see Fig. 4.1), depicting the interaction of replicase with the RNA segments derived from the internal binding sites (M-site and S-site), we now consider the binding of the enzyme to Qβ RNA under initiation conditions. Several different binding complexes are consistent with these models (Fig. 4.4).

(A): "Double loop" structure. Qβ RNA interacts at 2 sites on the enzyme ($B_{S,M}$). These two sites have a similar affinity for M-site and S-site.
(B),(C): Terminally looped structures. One site (B_{SM}) on the enzyme has a strong affinity for the internal regions on the RNA (S-site or M-site), structure (B), or 2 sites on the enzyme B_S and B_M. The binding at these two sites is mutually exclusive, structure (C).
Although it is not possible to distinguish between these models at present, electron microscopic studies of Qβ RNA-Qβ replicase binding complexes (work in progress), might allow the visualization and the quantitation of the binding complexes present.

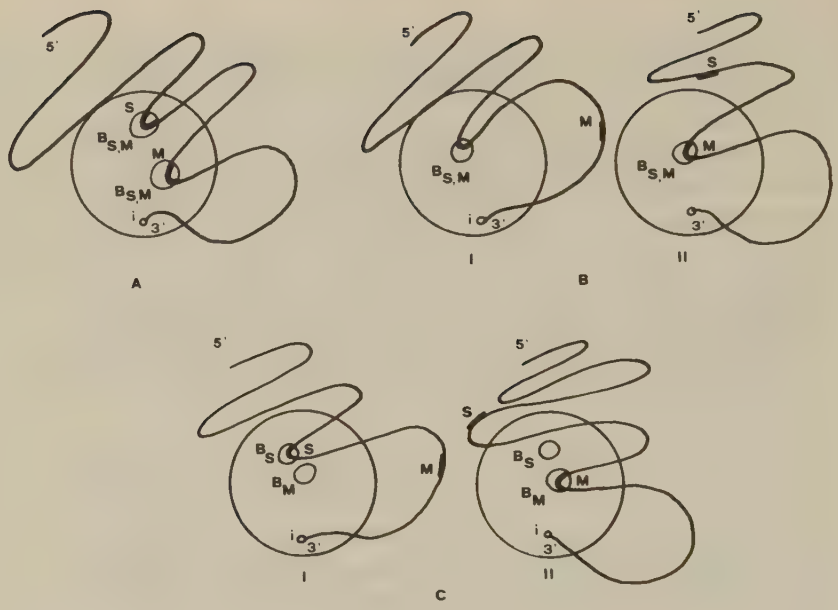

Figure 4.4 Possible structures of binding complexes obtained under initiation conditions.

Whatever model will turn out to be correct, it can be concluded that the way Qβ RNA is recognized by replicase is not through the binding at its 3'-end, where synthesis starts. Using different experimental approaches, the 3'-end per se was not found to bind to the enzyme under our assay conditions. Thus the interaction of replicase with the internal binding sites on Qβ RNA is the mechanism designed to enable the enzyme to find the 3'-end of its highly compact, structured natural template. We suggest that the binding to the M-site is required for the 3'-end binding and that the interaction at the S-site is probably only involved in the replicase repressor function in protein synthesis (Weber et al., 1972). The S-site RNA fragment S-3 interfered with the initiation of Qβ RNA synthesis

because both RNA fragments (M-site and S-site) interact at the same (or mutually exclusive) site(s) on the enzyme (Fig. 4.1, models b,c,d).
In order to promote a strong interaction of the 3'-end with the enzyme, both host factor I and GTP are required in addition. Host factor I may be essential for the folding of the RNA such that the 3'-end can be directed in the initiation site(i). Upon synthesis, when GTP is added, the 3'-end is strongly bound in site (i).

What questions do these models raise about the structure of the replicative intermediate? Does the enzyme become detached from the internal binding site(s) and/or the 3'-end after chain elongation begins? If so, at what stage during ongoing RNA synthesis does the dissociation occur? Additional evidence is required before we can answer these questions.

In this connection it may be noted that a different model for RNA phage replication has been proposed by H. Robertson (Zinder, 1975). This model does not rely on interactions between replicase and internal binding sites on the RNA, but assumes that the 3'-end of the template strand (whether it be plus or minus) and the 5'-end of the growing nascent strand are bound to replicase. During synthesis of the minus strand, the 3'-end of the plus strand and the 5'-end of the newly synthesized minus strand remain attached to the enzyme, resulting in a"butterfly" structure. In this model the annealing of the nascent strand to the template strand is prevented, a necessary feature of phage RNA synthesis since both the plus and the minus strand are synthesized in a single stranded structure (Weissmann et al., 1968).

5) Interaction between Qβ replicase and Qβ RNA in the presence of Mn^{2+}-ions

It had been shown previously that by substituting Mn^{2+}-ions for Mg^{2+}-ions in an in vitro reaction, Qβ replicase accepted Qβ RNA and a large variety of other RNA molecules as a template for the synthesis of the complementary strand (Palmenberg & Kaesberg, 1974; Obinata et al., 1975). This reaction was independent of host factor I and required GTP to initiate synthesis.

Binding experiments showed that generally, the binding of the enzyme to Qβ RNA was increased 3 to 4 fold compared to conditions where Mg^{2+} was present. The predominant interactions found were at the internal binding sites (M-site and S-site) and at the 3'-terminal region in addition. A selective stimulation of the binding of the 3'-terminus was found upon adding GTP to the reaction mixture (about 4 fold).

Binding replicase to T_1-digested Qβ RNA in the presence of Mn^{2+}-ions showed that the binding of the enzyme to the T_1 oligonucleotides was increased 2 to 10 fold in general, compared to conditions where Mg^{2+} or salt was present, however the binding specificity was reduced: Oligonucleotides showing insignificant binding under conditions where Mg^{2+} was present, were bound very efficiently to the enzyme in the presence of Mn^{2+}-ions. Furthermore, in contrast to what was found in the presence of Mg^{2+}, addition of GTP to the reaction mixture selectively increased the binding of the 3'-terminal oligonucleotide to the enzyme about 12 fold.

These results suggest that in the presence of Mn^{2+}, the binding affinity of the enzyme to RNA is increased but the specificity is thereby diminished. Addition of GTP promotes a strong interaction with the 3'-terminal oligonucleotide per se and therefore one might expect that in the presence of Mn^{2+} and GTP

the interaction at the 3'-terminus is sufficient for template binding and the interaction at the internal sites might be dispensable.

Concluding remarks

From the results of the present work the main conclusions in regard to template recognition by Qβ replicase can be summarized as follows: The recognition of Qβ RNA by Qβ replicase is not through the binding to the 3'-end of its template, where synthesis starts. The enzyme binds strongly to 2 internal sites on Qβ RNA (S-site and M-site) one of which (M-site) is required for template recognition. The interaction at these sites involves recognition of sequences rather than recognition of secondary and/or tertiary structural elements. The enzyme can selectively bind to sequences derived from the 3'-terminal region when the RNA structure is disrupted by complete digestion with RNase T_1, however in intact RNA these sites are only bound significantly when initiation of Qβ RNA synthesis occurs. We suggest that for the formation of a productive initiation complex a strong interaction at the M-site is required and as a consequence of this interaction due to the secondary and/or tertiary structure of the RNA the 3'-end of the RNA is placed at the enzyme initiation site, where it is weakly bound. In order to promote a strong interaction of the 3'-terminal region and the 3'-terminus with replicase both host factorI and GTP are required in addition.

The interactions which occur between replicase and its template seem to be a rather elaborate mechanism to assure specific template recognition. However a not less complex situation appears to hold for the interactions between aminoacyl tRNA synthetase and tRNA. The recognition of the tRNA by its synthetase is not restricted to an interaction at the 3'-OH end

of the tRNA, where aminoacylation occurs. Synthetase seems to recognize common features associated with the 3-dimensional structure of all tRNAs, whereas the detailed specificity is likely to lie in additional interactions involving the recognition of certain base sequences as for example in the acceptor stem or in the D stem.

In contrast to the wide range of interactions involving proteins and RNA there is a relatively narrow range of interactions found between proteins and DNA. Proteins such as restriction enzymes and the regulatory proteins, which determine the specificity of transcriptional events such as RNA polymerase and repressors, appear to recognize their sites by sequence selection only.

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APPENDIX

Details of the sequence determination of fragment M-2a*

1. RNase T_1 and RNase A digestion products of M-2a.

The data needed for establishing the oligonucleotide catalogs given in section 3.2 are compiled in Table A1 and Table A2.

Oligonucleotides obtained by RNase T_1 digestion were subjected to RNase A and RNase U_2 cleavage and base analysis was performed by combined RNase A and T_2 digestion. In some cases, as for the oligonucleotides T7 and T5 (see Fig. A-1) additional information was necessary. T5 was digested partially by RNase U_2 and T7 was digested by ribonuclease 1 from Physarum polycephalum (see Methods, section 2.2.5.a).

Similarly RNase A digestion products were recovered from the fingerprint and further analysed by RNase T_1 digestion and base analysis.

The T_1 oligonucleotides T2 and T27 (T27 is a mixture of 4 isomers) could not be completely determined using the approach described above. The data accumulated for T2 yielded the sequence AUUUCAAUU(U,C)UCACUG[G] with one ambiguity remaining. The sequence of this oligonucleotide was independently resolved and shown to be AUUUCAAUUUCUCACUG[G] (Billeter, M. A., unpublished results). For the sequences of the oligonucleotides T27, see below.

The information given in Table A2 was sufficient to yield unambiguous sequences for RNase A oligonucleotides, except for P1 and P3. Two possible sequences remained for P1, namely GAGGGU[A] or GGAGGU[A] . Nearest neighbor information

* Only the data for the fragment M-2a will be described subsequently. The sequence of the fragment M-2b will be discussed at the end of this section in relation to the sequence of M-2a.

from the partial digestion products of the fragment M-2a resolved this ambiguity. As explained below, the 5'-terminus of P1 must overlap with the 3'-end of T17, UUG[A] . P1 therefore has the sequence: GAGGGU[A] . Similarly two possibilties remained for P3: AG(G,A)GAU[G] . This ambiguity could be resolved by comparison to the complementary sequence TM18: GAACAUCUCCUAUUAG (determined by M.A.Billeter, unpublished results) as well as from the nearest neighbor information from the partial digestion products of the fragment M-2a (see Plate A, T23). Its sequence proved to be AGGAGAU[G] .

2. Partial digestion products.

The complete nucleotide sequence of M-2a was determined by using data from partial nuclease digestion yielding fragments of 20-100 nucleotides in length. Proceding from smaller to larger fragments, the oligonucleotides present in their corresponding RNase T_1 and RNase A fingerprints were ordered by considering the information given by the nearest neighbor of every oligonucleotide as well as the overlapping sequences between the RNase T_1 and RNase A oligonucleotides.

Partial digestion was carried out with RNase T_1 and carboxymethylated RNase A under several conditions and resulted in a mixture of RNA fragments which were fractionated by polyacrylamide gel electrophoresis (see Methods, section 2.2.5.b). The isolated fragments were analysed by small scale RNase T_1 and RNase A fingerprints, as described in the Methods section. The oligonucleotides were identified by their position on the fingerprints and/or by further analysis. Additional information was obtained by using α-^{32}P GMP-labeled fragment M-2a and M-2b prepared from RNA synthesized in vitro (see Methods, section 2.2.4) as substrate for partial digestion by RNase T_1. The RNase T_1 and RNase A oligonucleotide catalogs

of the partial digestion products used to establish the complete nucleotide sequence of the fragment M-2a, are summarized in Table A3. The information of Table A3 together with the two oligonucleotide catalogs of the fragment M-2a (Table 3.1 and Table 3.2) was sufficient to define an unambiguous sequence for the entire fragment M-2a.

The assembly was performed in discrete steps (see Plate A). The puzzle starts with the partial digestion product CM-Pa (12.2): Its unique sequence is shown in Plate A:CM-Pa(12.2). The partial digestion product T(23)which contains all the T_1 oligonucleotides present in CM-Pa(12.2) and in addition T9 and T26, has the unique sequence:T(23).This sequence resolves the ambiguity present in the RNase A oligonucleotide P3: AG(G,AG)AU[G] . The nearest neighbor of the T_1 oligonucleotide T9 proves the sequence: AGGAGAU[G] .

Determination of the sequence of the partial digestion product CM-Pa(11):

The nucleotide sequence of T27d present in this fragment, was established by digestion of T27 by ribonuclease from Physarum polycephalum, as described in Methods, section 2.2.5.a. The main digestion product was found to be CCG (no CCU, CG and CU was found), which defined the sequence: UCCG[A/U].

CM-Pa(11) starts at its 5'-end with the RNase A oligonucleotide P3, which is located at the 3'-end of T(23). P3, overlapping with T26 defines the location of T26.

The T_1 oligonucleotide TM18 (determined by M. A. Billeter, unpublished results) is complementary to the sequence from T9 (see partial product T(23)) to T26 (see 5'-end of CM-Pa(11)) including the first 3 nucleotides of T16. This overlap locates T16 next to T26. T16 is followed by T27d and not by T17, because T17 overlaps with the 5'-end of P1, which has to be at the 3'-end of CM-Pa(11).

The partial digestion product 12(G-label) comprises all oligo-

nucleotides of CM-Pa(12.2) (except its 5'-end: T35) and CM-Pa(11) and contains in addition the T_1 oligonucleotides T15, T17, T37a and T9a and the RNase A products P18, P12, P19 and P22, which can be only arranged in a unique sequence. The sequence of the fragment 12(GTP-label) could be confirmed in addition by the T_1 oligonucleotide TM24, which is complementary to the 3'-end of the fragment 12(GTP-label). The nucleotide sequence of TM24 has been established by M. A. Billeter, unpublished results.

The partial digestion product T(32), has the unique sequence: T(32).

The RNase T_1 and RNase A products of the partial digestion product b(P/P) uniquely define the sequence b(P/P).

The oligonucleotides of the fragment d(P/P) can be arranged in two different sequences, d(P/P):a and d(P/P):b. The nucleotide sequence of T27c in fragment d(P/P) was established by digestion of T27 from the partial digestion product CM-Pa(8) (see Fig. A5) with ribonuclease from Physarum polycephalum (see Methods, section 2.2.5.a). The main digestion products were found to be CG and CU (no CCG and CCU were detected), which defined the sequence to be CUCG[U].

The partial digestion product 12(P/P) comprises all the oligonucleotides present in b(P/P) and d(P/P) and contains in addition the RNase A product GGGC (P7) and therefore has the structure 12(P/P):a or 12(P/P):b. The structure 12(P/P):b and consequently d(P/P):b could be excluded by the sequence of the partial digestion product CM-Pa(7), which contains all the oligonucleotides of b(P/P) and in addition the T_1 oligonucleotides T27, T35 at its 5'-end and T37, T35 and T14 at its 3'-end, but not T5. Furthermore 12(P/P):a predicts one mole of CG (T37) followed by A, which could be confirmed by the partial digestion product T2d(ATP-label) (see Table A3 and Fig. A5). The nucleotide sequence of T27b in fragment CM-Pa(7) was determined by digestion of T27 from the partial digestion

product CM-Pa(12.1) (see Fig. A5) with ribonuclease from Physarum polycephalum. The trinucleotide CCU, as the main digestion product, proves the sequence CCUG. In fragment CM-Pa(7), T27b has to be followed by A.

The partial digestion product T(7) comprises all the sequences present in fragment 12(P/P):a and contains 2 moles of T27 and one mole each of T4 and T32, in addition. It contains furthermore the RNase A products P9, P17, P19 and P20 which are absent in fragment 12(P/P):a.

P17 and P9 extend the sequence at the 5'-end of the fragment 12(P/P):a (see fragment CM-Pa(7)). Furthermore T4 has to be at the 5'-end of T27b because the 3'-end of T4 overlaps with P9. The oligonucleotides T32 and T27a are located at the 5'-end of the fragment T(7) and not at its 3'-end for the following reasons:

1) This arrangement would give rise to the RNase A product AGAAC, which was not found in the RNase A oligonucleotide catalog of the RNA fragment M-2a (Table 3.2).

2) There are two -PyAGA- oligonucleotides in the RNA fragment M-2a (Table 3.2): P5: AGAGGC U and P4: AGAAAAAU.

P5 has already been located unambiguously in fragment T(32). The only possible oligonucleotide, which can elongate the 3'-end of the fragment T(7) is therefore the T_1 oligonucleotide AAAAAUAG[C](T11), located at the 5'-end of the partial digestion product 12(GTP-label). This arrangement links the 3'-end of the fragment T(7) to the 5'-end of the fragment 12(GTP-label).

The nucleotide sequence of T27a was established by digesting T27 from the partial digestion product CM-Pa(9) (see Fig. A5) with ribonuclease from Physarum polycephalum. The main digestion products were found to be CU and CG. Therefore its sequence has to be CUCG[U].

The partial digestion product 1(P/P) comprises all the oligonucleotides present in fragment T(7) and contains in addition T6, which must be located at its 5'-end.

The partial digestion product T(4), which contains in addition to all the oligonucleotides present in 1(P/P) the RNase T_1 products T30, T23 and T29 extends the sequence of the fragment 1(P/P) at its 5'-end.
The 3'-end of the fragment T(32) can be linked to the 5'-end of the fragment T(4) because T29, which is located at the 3'-end of the fragment T(32), is also present at the 5'-end of the fragment T(4). The overlapping oligonucleotide T29 establishes the linkage between the two fragments.
It can be concluded that the 5'-end of the partial digestion product 12(GTP-label) must be linked to the 3'-end of the partial digestion product T(7), as discussed above, and consequently to the 3'-end of the partial digestion product T(4). The 3'-end of the fragment T(32) overlaps with the 5'-end of the fragment T(4).This arrangement covers all the RNase T_1 and RNase A products present in the RNA fragment M-2a.
The complete nucleotide sequence of the intact RNA fragment M-2b is shown in Fig. A5. Other partial digestion products, which have not been discussed in detail, were found to be consistent with the proposed sequence.

3. Nucleotide sequence of the RNA fragment M-2b.

As can be seen in Fig. 3.2., fragment M-2b moved under non-denaturing conditions faster than M-2a on a 20% polyacrylamide gel. However under denaturing conditions on a 15% polyacrylamide gel in 7 M urea, the relative mobility of the two RNA bands was reversed (data not shown). This suggests that M-2b is in fact larger in size than M-2a.
Partial digestion of the fragments M-2a and M-2b by RNase T_1 showed one remarkable difference in the fragment pattern obtained upon fractionation of the digest on a 20% polyacrylamide gel (data not shown). In the digest obtained from the fragment M-2b there was an additional band present, which was absent (or only present in background amounts) in the pattern

derived from M-2a. This band called partial digestion product 12(GTP-label) (see Table A3), contained the 3'-end of M-2a including the terminal T_1 oligonucleotide CG[U], as well as the expected RNase A product GC[G]. In addition CCG (T37a, Fig. A2) and UCACUG (T9a, Fig. A2) were present; T9a was not clearly resolved from T9. In the RNase A fingerprint, no additional characteristic spot could be expected, except for an additional C[G] and U[G].

The quantitation and the characterisation of the oligonucleotides T37a and T9a were performed furthermore by analysing the fingerprints obtained from the purified fragment M-2b and a mixture containing both fragments M-2a and M-2b. The results are summarized in Table A4.

The RNase A digestion products of M-2a and M-2b were qualitatively the same (see Fig. A3 and A4). However by comparing the molar yields of the oligonucleotides P12, P19, P22, P23 and P24, significant quantitative differences between M-2a and M-2b were found in the RNase A fingerprints. Compared to M-2a M-2b contained one mole of P12 (GU), P22 (AC) and 1.7 mole of P19 (GC) in addition.

From these data it was concluded that compared to M-2a, fragment M-2b, at its 3'-end, carries the following additional sequence of 9 nucleotides: M-2a + UCACUGCCG[A] (see Fig. A5).

The fragments M-2c and M-2d, indicated in Fig. 3.2. were not analysed systematically, because they did not appear reproducibly enough. However the oligonucleotides present in their respective fingerprints indicated that they were derived from the fragment M-2b.

Plate A

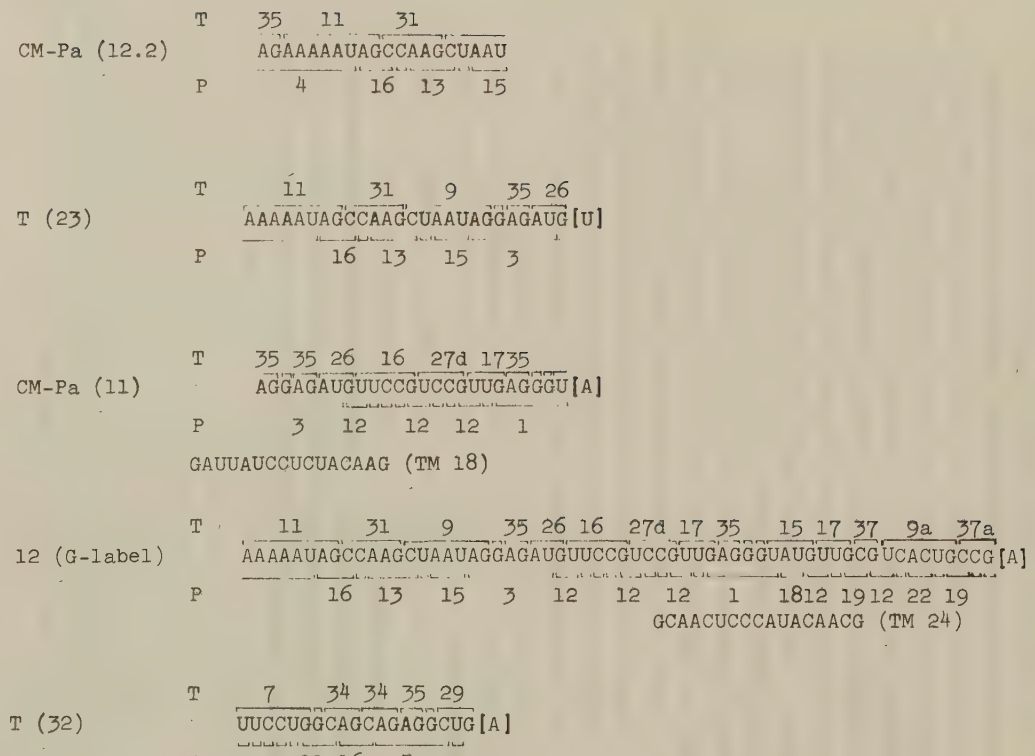

T 35 11 31
CM-Pa (12.2)
AGAAAAAUAGCCAAGCUAAU
P 4 16 13 15
T 11 31 9 35 26
T (23)
AAAAAUAGCCAAGCUAAUAGGAGAUG[U]
P 16 13 15 3
T 35 35 26 16 27d 1735
CM-Pa (11)
AGGAGAUGUUCCGUCCGUUGAGGGU[A]
P 3 12 12 12 1
GAUUAUCCUCUACAAG (TM 18)
T 11 31 9 35 26 16 27d 17 35 15 17 37 9a 37a
12 (G-label)
AAAAAUAGCCAAGCUAAUAGGAGAUGUUCCGUCCGUUGAGGGUAUGUUGCGUCACUGCCG[A]
P 16 13 15 3 12 12 12 1 1812 1912 22 19
GCAACUCCCAUACAACG (TM 24)
T 7 34 34 35 29
T (32)
UUCCUGGCAGCAGAGGCUG[A]
P 11 16 5

Plate A

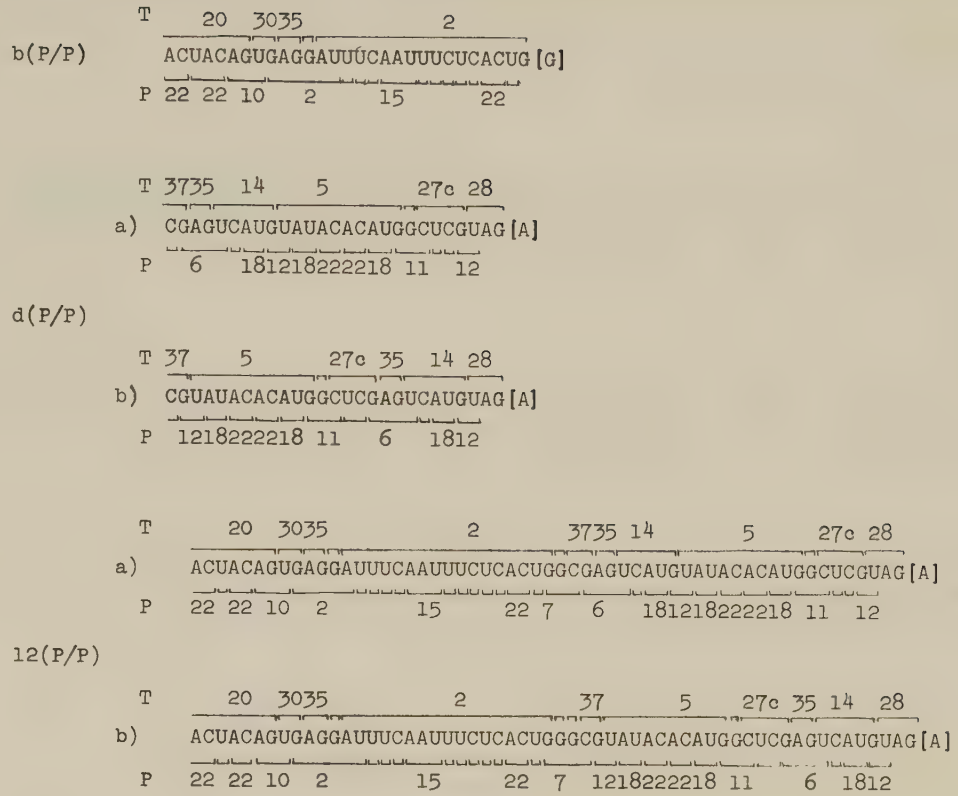
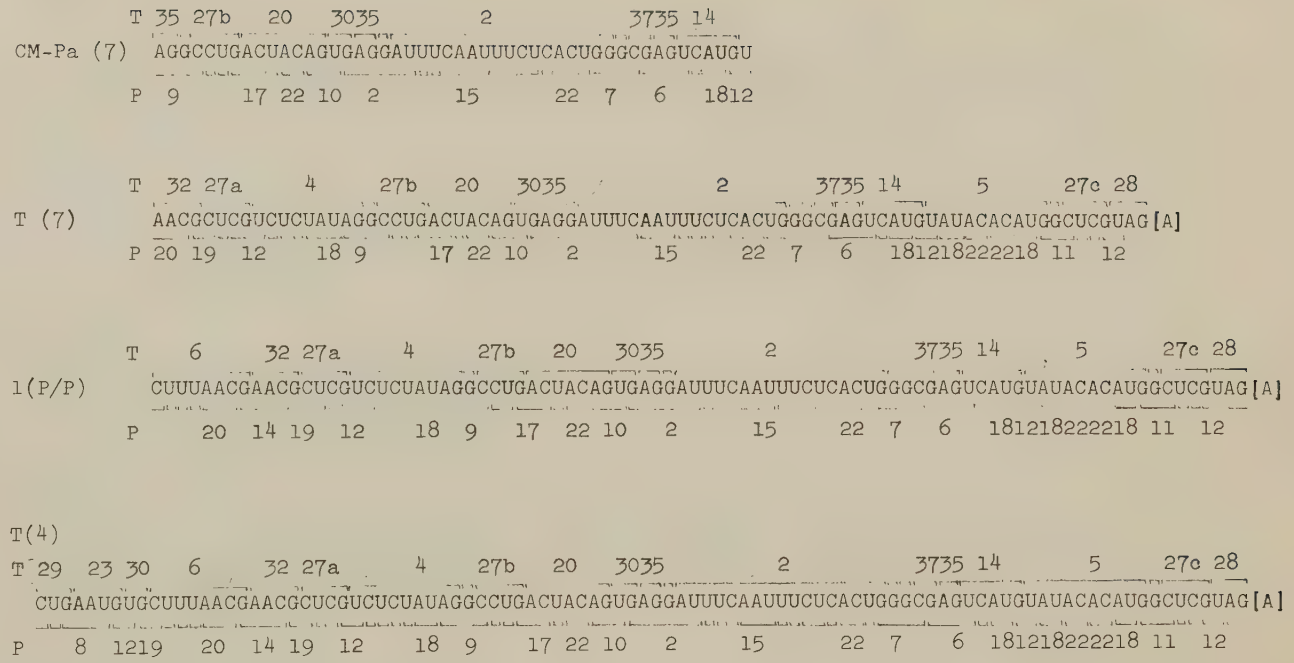

T 20 3035 2
b(P/P) ACUACAGUGAGGAUUUCAAUUUCUCACUG[G]
P 22 22 10 2 15 22
T 3735 14 5 27c 28
a) CGAGUCAUGUAUACACAUGGCUCGUAG[A]
P 6 1812182222218 11 12
d(P/P)
T 37 5 27c 35 14 28
b) CGUAUACACAUGGCUCGAGUCAUGUAG[A]
P 1218222218 11 6 1812
T 20 3035 2 3735 14 5 27c 28
a) ACUACAGUGAGGAUUUCAAUUUCUCACUGGCGAGUCAUGUAUACACAUGGCUCGUAG[A]
P 22 22 10 2 15 22 7 6 1812182222218 11 12
12(P/P)
T 20 3035 2 37 5 27c 35 14 28
b) ACUACAGUGAGGAUUUCAAUUUCUCACUGGGCGUAUACACAUGGCUCGAGUCAUGUAG[A]
P 22 22 10 2 15 22 7 1218222218 11 6 1812

Plate A


T 35 27b 20 3035 2 3735 14
CM-Pa (7) AGGCCUGACUACAGUGAGGAUUUCAAUUUCUCACUGGGCGAGUCAUGU
P 9 17 22 10 2 15 22 7 6 1812
T 32 27a 4 27b 20 3035 2 3735 14 5 27c 28
T (7) AACGCUCGUCUCUAUAGGCCUGACUACAGUGAGGAUUUCAAUUUCUCACUGGGCGAGUCAUGUAUACACAUGGCUCGUAG[A]
P 20 19 12 18 9 17 22 10 2 15 22 7 6 181218222218 11 12
T 6 32 27a 4 27b 20 3035 2 3735 14 5 27c 28
1(P/P) CUUUAACGAACGCUCGUCUCUAUAGGCCUGACUACAGUGAGGAUUUCAAUUUCUCACUGGGCGAGUCAUGUAUACACAUGGCUCGUAG[A]
P 20 14 19 12 18 9 17 22 10 2 15 22 7 6 181218222218 11 12
T(4)
T 29 23 30 6 32 27a 4 27b 20 3035 2 3735 14 5 27c 28
CUGAAUGUGCUUUAACGAACGCUCGUCUCUAUAGGCCUGACUACAGUGAGGAUUUCAAUUUCUCACUGGGCGAGUCAUGUAUACACAUGGCUCGUAG[A]
P 8 1219 20 14 19 12 18 9 17 22 10 2 15 22 7 6 181218222218 11 12

Footnote to Plate A

T stands for RNase T_1 products; P stands for RNase A products. The numbers indicated above and below the nucleotide sequence correspond to the numbers of the digestion products, indicated in Fig. A-1 to A-4. Mononucleotides are not indicated.

Table A1 Sequence of T1 oligonucleotides indicated in Fig. A1

Products of T_1 oligonucleotides derived from homogeneously labeled RNA.

Oligo-nucleotide	Sequence[1]	Molar yield[2]	RNase T_2 products	RNase A products	RNase U_2 products
T2	AUUUCA$_2$UU(U,C)UCACUG[G]	0.7	1 G	1 G	0.6 $(U_4,C_2)A$[a]
			4.2 C	1.2 AAU	1.1 $(U_3,C)A$[b]
			3.8 A	1.4 AU	0.7 (U,C)G
			8.4 U	0.6 AC	2 A
				2.8 C	
				5.7 U	

Products of T_1 oligonucleotides from RNA synthesized in vitro in presence of one α-^{32}P-nucleoside triphosphate.

GTP-label[9]		ATP-label			UTP-label[9]			CTP-label[9]	
^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	RNase A products	RNase U_2 products	^{32}P equiv.[3]	RNase A products	RNase U_2 products	^{32}P equiv.[3]	RNase U_2 products
2.0	1 U*	2.2	1 A*AU	$U_3C^*A^*$	5.9	1 C*	1 C*UG	3.6	1 UUU*CA
	1.0 G*		1.9 C*	$(U_4,C_2)A$		1.0 AA*U*	0.5 (2U*,2U,C*)CA		0.7 (2U,2U*,C)CA*
						1.1 A*U*	1.5 2U*UCA		
						0.7 AC*			
						1.7 U*			

Table A1 (continued)

Products of T_1 oligonucleotides derived from homogeneously labeled RNA.

Oligonucleotide	Sequence[1]	Molar yield[2]	RNase T_2 products	RNase A products	RNase U_2 products
T4	UCUCUAUAG[G]	1.0	1 G	1 AG	1.0 $(U_3C_2)A$
			2.3 C	1.2 AU	1.0 UA
			1.9 A	2.3 C	1 G
			4.0 U	3.5 U	

Products of T_1 oligonucleotides from RNA synthesized in vitro in presence of one α-^{32}P-nucleoside triphosphate

GTP-label[9]			ATP-label			UTP-label[9]			CTP-label[9]	
^{32}P equiv.[3]	RNase T_2 products	RNase A products	^{32}P equiv.[3]	RNase A products	RNase U_2 products	^{32}P equiv.[3]	RNase T_2 products	RNase A products	^{32}P equiv.[3]	RNase T_2 products
2.0	1 A*	A*G*	2.6	1 U*	1 $(U_2,C_2)U^*A$	3.6	1 A*	1 A*U	2.6	U*
	0.9 G*			1.0 AU*	1.2 U*A		1.8 C*	1.5 C*		
							0.5 U*			

Table A1 (continued)

Products of T_1 oligonucleotides derived from homogeneously labeled RNA.

Oligonucleotide	Sequence[1]	Molar yield[2]	RNase T_2 products	RNase A products	RNase U_2 products	Partial digestion with RNase U_2 some products[4]
T5	UAUACACAUG[G]	0.8	1 G	1 G	1 UG	UAUA
			2.3 C	2.2 AU	2.0 UA	CAUG
			3.7 A	2.0 AC	2.0 CA	CACAUG
			3.3 U	1.2 U		UAUACA

Products of T_1 oligonucleotides from RNA synthesized in vitro in presence of one α-^{32}P-nucleoside triphosphate.

GTP-label[9]			ATP-label			UTP-label[9]			CTP-label[9]	
^{32}P equiv.[3]	RNase T_2 products	RNase A products[c]	^{32}P equiv.[3]	RNase A products	RNase U_2 products	^{32}P equiv.[3]	RNase T_2 products	RNase U_2 products	^{32}P equiv.[3]	RNase A products
1.7	1 U*	G*	2.0	1 U*	1 C*A	1.9	1 A*	1 UA*	1.9	A*C
	0.9 G*	AU*		0.9 AU*	0.7 U*A		0.1 U*	1.0 CA*		
				1.5 AC*						

Table A1 (continued)

Products of T_1 oligonucleotides derived from homogeneously labeled RNA.

Oligonucleotide[5]	Sequence[1]	Molar yield[2]	RNase T_2 products	RNase A products	RNase U_2 products
T6	CUUUAACG[A]	0.6	2 A	1 G	1.2 $(U_3,C)A$
			0.7 G	1.1 AAC	1.4 A
			2.4 C	1.2 C	1 CG
			3.5 U	3.2 U	

Products of T_1 oligonucleotides from RNA synthesized in vitro in presence of one α-^{32}P-nucleoside triphosphate.

GTP-label[9]			ATP-label		UTP-label[9]		CTP-label[9]	
^{32}P equiv.[3]	RNase T_2 products	RNase A products	^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	RNase A products
0.8	C*	AAC*	1.3	1 U*	1.5	1 C*	0.6	AA*C
				0.9 G*		2.0 U*		

Table A1 (continued)

Products of T_1 oligonucleotides derived from homogeneously labeled RNA.

Oligonucleotide	Sequence[1]	Molar yield[2]	RNase T_2 products	RNase A products	RNase 1 from Ph.PC. some products[4]
T7	UUCCUG[G]	1.0	1 G	1 G	CCUG
			2.2 C	1.4 C	CCU
			2.9 U	2.6 U	UUCCU

Products of T_1 oligonucleotides from RNA synthesized in vitro in presence of one α-^{32}P-nucleoside triphosphate.

GTP-label[9]			UTP-label[9]			CTP-label[9]		ATP-label
^{32}P equiv.[3]	RNase T_2 products	RNase A products[c]	^{32}P equiv.[3]	RNase T_2 products	RNase A products	^{32}P equiv.[3]	RNase T_2 products	not present
1.7	1 U*	G*	2.0	1 U*	1 U*	1.8	1 U*	
	0.8 G*	U*		0.9 C*	1.1 C*		0.7 C*	

Table A1 (continued)

Oligonucleotide	Sequence[1]	Molar yield[2]	RNase T_2 products	RNase A products	RNase U_2 products
T9	CUAAUAG[G]	0.8	1 G	1 AG	1.0 (U,C)A
			1.3 C	1.1 AAU	1.0 UA
			3.2 A	0.8 C	1.3 A
			1.5 U	0.9 U	1 G

Products of T_1 oligonucleotides from RNA synthesized in vitro in presence of one α-^{32}P-nucleoside triphosphate.

GTP-label[9]			ATP-label			UTP-label[9]		CTP-label[9]
^{32}P equiv.[3]	RNase T_2 products	RNase A products	^{32}P equiv.[3]	RNase A products	RNase U_2 products	^{32}P equiv.[3]	RNase A products	not present
2.2	1 A*	A*G*	2.6	1 U*	UA	1.9	1 AA*U	
	0.9 G*			1.0 A*AU*	(C,U)A		1.1 C*	

Table A1 (continued)

Products of T_1 oligonucleotides derived from homogeneously labeled RNA.

Oligonucleotide	Sequence[1]	Molar yield[2]	RNase T_2 products	RNase A products	RNase U_2 products
T11	AAAAAUAG[C]	0.6	1 G 0.3 C 6.9 A 1.5 U	1 AG 1.4 A_5U[f]	1 G 4.2 A 0.8 UA

Products of T_1 oligonucleotides from RNA synthesized in vitro in presence of one α-^{32}P-nucleoside triphosphate.

GTP-label[9]		ATP-label		UTP-label[9]		CTP-label[9]	
^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	RNase A products	^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	RNase T_2 products
0.7	A*	2.2	A*A*A*A*AU*	0.8	1 A* 0.1 C*	0.8	G*

Table A1 (continued)

Products of T_1 oligonucleotides derived from homogeneously labeled RNA.

Oligonucleotide	Sequence[1]	Molar yield[2]	RNase T_2 products	RNase A products	RNase U_2 products
T14	UCAUG[U]	1.0	1 G	1 G	1 UG
			0.8 C	1.0 AU	1.0 (U,C)A
			0.9 A	1.1 C	
			1.8 U	1.0 U	

Products of T_1 oligonucleotides from RNA synthesized in vitro in presence of one α-^{32}P-nucleoside triphosphate.

GTP-label[9]		ATP-label		UTP-label[9]		CTP-label[9]	
^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	RNase T_2 products
1.0	U*	1.3	C*	2.2	1 A* 1.0 G*	1.0	U*

Table A1 (continued)

Products of T_1 oligonucleotides derived from homogeneously labeled RNA.

Oligonucleotide	Sequence[1]	Molar yield[2]	RNase T_2 products	RNase A products	RNase U_2 products
T15	UAUG[U]	1.0	1 G 0.8 A 1.5 U	1 G 1.2 AU 1.0 U	1 UG 0.9 UA

Products of T_1 oligonucleotides from RNA synthesized in vitro in presence of one α-^{32}P-nucleoside triphosphate.

GTP-label[9]		ATP-label		UTP-label[9]		CTP-label[9]
^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	RNase T_2 products	not present
1.0	U*	1.3	U*	1.7	1 G* 1.0 A*	

Table A1 (continued)

Products of T_1 oligonucleotides derived from homogeneously labeled RNA.

Oligonucleotide	Sequence[1]	Molar yield[2]	RNase T_2 products	RNase A products
T16	UUCCG[U]	1.0	1 G	1 G
			1.7 C	1.6 C
			1.7 U	1.9 U

Products of T_1 oligonucleotides from RNA synthesized in vitro in presence of one α-^{32}P-nucleoside triphosphate.

GTP-label[9]			UTP-label[9]		CTP-label[9]		ATP-label
^{32}P equiv.[3]	RNase T_2 products	RNase A products	^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	RNase T_2 products	not present
0.9	C*	C*	2.6	1 G*	2.1	1 U*	
				1.0 U*		0.9 C*	

Table A1 (continued)

Products of T_1 oligonucleotides derived from homogeneously labeled RNA.

Oligonucleotide	Sequence[1]	Molar yield[2]	RNase T_2 products	RNase A products
T17	UUG[A]	2.2	1 G	1 G
	UUG[C]		2.0 U	2.0 U

Products of T_1 oligonucleotides from RNA synthesized in vitro in presence of one α-^{32}P-nucleoside triphosphate.

GTP-label[9]		ATP-label	UTP-label[9]		CTP-label[9]	
^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	RNase T_2 products
2.1	U*	1.5	2.1	U*	0.9	G*

Table A1 (continued)

Products of T_1 oligonucleotides derived from homogeneously labeled RNA.

Oligonucleotide	Sequence[1]	Molar yield[2]	RNase T_2 products	RNase A products	
T20	ACUACAG[U]	0.7	1 G	1 AG	0.7 (U,C)A
			2.1 C	2.0 AC	0.8 CA
			2.2 A	1.0 U	0.7 A
			0.9 U		1 G

Products of T_1 oligonucleotides from RNA synthesized in vitro in presence of one α-^{32}P-nucleoside triphosphate.

GTP-label[9]		ATP-label			UTP-label[9]		CTP-label[9]	
^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	RNase A products	RNase U_2 products	^{32}P equiv.[3]	RNase A products	^{32}P equiv.[3]	RNase U_2 products[c]
0.9	A*	1.0	1 AC*	1 CU*A	1.3	1 AC*	1.8	(C,U)A*
			1.2 U*	1.0 C*A		1.4 AG*		A*
			0.3 AG*					

Table A1 (continued)

Products of T_1 oligonucleotides derived from homogeneously labeled RNA.

Oligonucleotide	Sequence[1]	Molar yield[2]	RNase T_2 products	RNase A products	RNase U_2 products
T23	AAUG[U]	1.1	1 G 0.1 C 2.3 A 1.2 U	1 G 1.1 AAU	1 UG 2.0 A

Products of T_1 oligonucleotides from RNA synthesized in vitro in presence of one α-^{32}P-nucleoside triphosphate.

GTP-label[9]			ATP-label		UTP-label[9]		GTP-label[9]
^{32}P equiv.[3]	RNase T_2 products	RNase A products	^{32}P equiv.[3]	RNase A products	^{32}P equiv.[3]	RNase A products	not present
1.0	U*	AAU*	1.3	1 A*AU 0.3 AU*	2.0	1 G* 1.4 AA*U 0.4 A*U	

Table A1 (continued)

Oligo-nucleotide	Sequence[1]	Uniform label: Molar yield[2]	Uniform label: RNase T_2 products	Uniform label: RNase A products	GTP-label[9]: ^{32}P equiv.[3]	GTP-label[9]: RNase T_2 products	UTP-label[9]: ^{32}P equiv.[3]	UTP-label[9]: RNase A products	CTP-label[9] ATP-label not present
T26	AUG[U]	1.1	1 G 0.1 C 1.3 A 1.4 U	1 G 1.2 AU	0.9	U*	2.9	1 G* 1.1 A* 0.1 C*	

Table A1 (continued)

Products of T_1 oligonucleotides derived from homogeneously labeled RNA.

Oligonucleotide	Sequence[1]	Molar yield[2]	RNase T_2 products	RNase A products
T27	(U,2C)G[U]	4.1	1 G	1 G
	(U,2C)G[U]		2.2 C	2.0 C
	(U,2C)G[U]		1.1 U	1.0 U
	(U,2C)G[A]			

Products of T_1 oligonucleotides from RNA synthesized in vitro in presence of one α-^{32}P-nucleoside triphosphate.

GTP-label[9]			ATP-label	UTP-label[9]		CTP-label[9]	
^{32}P equiv.[3]	RNase T_2 products	RNase A products[c]	^{32}P equiv.[3]	^{32}P equiv.[3]	RNase A products	^{32}P equiv.[3]	RNase T_2 products
3.9	1 U*	U*	1.3	6.7	3 G*	5.2	2 C*
	3.1 C*	C*			4.1 C*		3.2 U*

Table A1 (continued)

Oligo-nucleotide	Sequence[1]	Uniform label: Molar yield[2]	Uniform label: RNase T_2 products	Uniform label: RNase A products	GTP-label[9]: ^{32}P equiv.[3]	GTP-label[9]: RNase T_2 products	ATP-label: ^{32}P equiv.[3]	ATP-label: RNase T_2 products	CTP-label[9] UTP-label[9] not present
T28	UAG[A]	1.0	1 G 0.4 C 0.6 A 0.9 U	1 AG 1.0 U	1.1	A*	2.7	1 U* 1.0 G*	

Table A1 (continued)

Oligo-nucleotide	Sequence[1]	Uniform label: Molar yield[2]	Uniform label: RNase T_2 products	Uniform label: RNase A products	GTP-label[9]: ^{32}P equiv[3]	GTP-label[9]: RNase T_2 products	GTP-label[9]: RNase A products	ATP-label[9]: ^{32}P equiv[3]	UTP-label[9]: ^{32}P equiv[3]	CTP-label[9]: ^{32}P equiv[3]
T29	CUG[A]	1.3	1 G 1.0 C 0.9 U	1 G 1.3 C 0.9 U	1.2	U*	U*	1.5	1.8	0.2

Oligo-nucleotide	Sequence[1]	Uniform label: Molar yield[2]	Uniform label: RNase T_2 products	Uniform label: RNase A products	GTP-label[9]: ^{32}P equiv[3]	GTP-label[9]: RNase T_2 products	ATP-label: ^{32}P equiv[3]	CTP-label[9]: ^{32}P equiv[3]	CTP-label[9]: RNase T_2 products	UTP-label[9] not present
T30	UG[A] UG[C]	2.3	1 G 1.0 U	1 G 1.3 U	2.0	U*	1.8	1.1	G*	

Oligo-nucleotide	Sequence[1]	Uniform label: Molar yield[2]	Uniform label: RNase T_2 products	Uniform label: RNase A products	GTP-label[9]: ^{32}P equiv[3]	GTP-label[9]: RNase T_2 products	ATP-label: ^{32}P equiv[3]	ATP-label: RNase T_2 products	CTP-label[9]: ^{32}P equiv[3]	CTP-label[9]: RNase A products	UTP-label[9] not pres.
T31	CCAAG[C]	0.8	1 G 1.8 C 1.9 A 0.2 U	1 AAG 1.7 C	0.8	A*	1.1	1 A* 1.0 C* 0.1 U*	1.3	1 AAG* 0.8 C*	

Table A1 (continued)

Oligonucleotide	Sequence[1]	Molar yield[2]	Uniform label: RNase T_2 products	Uniform label: RNase A products	GTP-label[9]: ^{32}P equiv[3]	GTP-label[9]: RNase T_2 products	GTP-label[9]: RNase A products	ATP-label: ^{32}P equiv[3]	CTP-label[9]: ^{32}P equiv[3]	CTP-label[9]: RNase A products	UTP-label[9] not pres.
T32	AACG[C][5]	0.4	1 G 1.1 C 2.2 A 0.2 U	1 G 1.2 AAC	0.8	C*	AAC*	0[6]	1.1	1 AA*C 0.7 G*	

Oligonucleotide	Sequence[1]	Molar yield[2]	Uniform label: RNase T_2 products	Uniform label: RNase A products	GTP-label[9]: ^{32}P equiv[3]	GTP-label[9]: RNase T_2 products	ATP-label: ^{32}P equiv[3]	ATP-label: RNase T_2 products	CTP-label[9]: ^{32}P equiv[3]	CTP-label[9]: RNase A products	UTP-label[9] not pres.
T34	CAG[C] CAG[A]	2.0	1 G 1.2 C 0.9 A	1 AG 0.9 C	1.9	A*	3.6	1 G* 2.0 C* 0.3 A* 0.1 U*	1.0	1 AG* 0.2 A*C	

Oligonucleotide	Sequence[1,8]	Molar yield[2]	Uniform label: RNase T_2 products	Uniform label: RNase A products	GTP-label[9]: ^{32}P equiv[3]	GTP-label[9]: RNase T_2 products	ATP-label: ^{32}P equiv[3]	UTP-label[9]: ^{32}P equiv[3]	CTP-label[9]: ^{32}P equiv[3]	CTP-label[9]: RNase A products
T35	(3) AG[G] (1) AG[A] (1) AG[U]	6.0	1 G 1.2 A	AG	7.3	3 G* 5.7 A*	1.8	1.5	0.3	AG*

Table A1 (continued)

Oligo-nucleotide	Sequence[1]	Uniform label: Molar yield[2]	Uniform label: RNase T_2 products	Uniform label: RNase A products	GTP-label[9]: ^{32}P equiv.[3]	GTP-label[9]: RNase T_2 products	ATP-label: ^{32}P equiv.[3]	UTP-label[9]: ^{32}P equiv.[3]	CTP-label[9]: ^{32}P equiv.[3]	CTP-label[9]: RNase T_2 products
T37[7]	CG[U] CG[A]	2.6	1 G 1.2 C	1 G 1.4 C	1.7	C*	2.7	0.9	0.5	G*

Nucleotide[1,e]		Uniform label: Molar yield[2]	Uniform label: RNase T_2 products	Uniform label: RNase A products	GTP-label[9]: ^{32}P equiv.[3]	ATP-label: ^{32}P equiv.[3]	UTP-label[9]: ^{32}P equiv.[3]	CTP-label[9]: ^{32}P equiv.[3]
T39	(2) G[G] (2) G[A] (5) G[C] (1) G[U]	10.0	G	G	1.5	2.8	1.6	5.5

Table A2 Sequence of Panc oligonucleotides indicated in fig. A3.

		Uniform label			GTP-label[9]			ATP-label			UTP-label[9]			CTP-label[9]	
Oligo-nucleotide	Sequence[1]	Molar yield[2]	RNase T_1 products	^{32}P equiv.[3]	RNase T_2 products	RNase T_1 products	^{32}P equiv.[3]	RNase T_2 products	RNase T_1 products	^{32}P equiv.[3]	RNase T_2 products	RNase T_1 products	^{32}P equiv.[3]	RNase T_1 products
P1	G(AG,G)GU[A]	0.7	1 U 3.4 G 1.1 AG	2.7	1 A* 1.9 G*	G* 1.0 A*G*	1.3	1 U* 1.1 G* 0.1 A* 0.1 C*		1.7	1 G* 0.3 U* 0.2 A* 0.3 C*	G*	absent	
P2	GAGGAU[U]	1.2	1 AU 1.9 G 0.9 AG	2.4	1 A* 1.0 G*	A*G*	1.7		G*	2.2	1 U* 1.0 A* 0.1 C*	A*U*	absent	
P3	AG(G,AG)AU[G]	0.9	1 AU 0.8 G 1.7 AG	4.4	1 U* 1.0 G* 1.9 A*	1 U* 1.0 A*G* 1.0 A*G	1.7		G* AG*	0.8	1 A* 0.1 C*		absent	
P4	AGAAAAAU[A]	0.9	1 AAAAAU 0.9 AG	1.0	A*		5.3		AAAAAU AG*	0.9	A*		absent	
P5	AGAGGC[U]	0.9	1 G 1.2 C 1.7 AG	2.9	1 G* 2.0 A*		0.9		AG*	0.7	1 C* 0.1 G*		0.7	G*

Table A2 (continued)

			Uniform label		GTP-label[9]		ATP-label	UTP-label[9]		CTP-label[9]	
Oligo-nucleotide	Sequence	Molar yield[2]	RNase T_1 products	^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	^{32}P equiv.[3]	RNase T_2 products	RNase T_1 products
P6	GAGU[C]	1.1	1 U 1.1 G 0.9 AG	1.2	A*	0.9	G*	1.0	0.9	U*	
P7	GGGC[G]	0.9	1 C 3.0 G	2.7	1 C* 1.9 G*	absent		absent	0.7	G*	
P8	GAAU[G]	1.0	1 AAU 1.0 G	1.0	U*	2.0	1 G* 1.1 A* 0.1 U*	1.0	absent		
P9	AGGC[C]	1.0	1 C 1.0 G 1.1 AG	2.4	1 A* 1.0 G*	absent		0.1	1.9		1 C* 1.1 G*
P10	AGU[G]	1.1	1 U 1.1 AG	2.2	1 U* 1.2 A*	absent		1.0	absent		
P11	GGC[A] GGC[U]	1.9	1 U 1.0 G	2.2	G*	0.9	C*	0.8	1.7	G*	

Table A2 (continued)

		Uniform label		GTP-label[9]		ATP-label		UTP-label[9]		CTP-label[9]	
Oligo-nucleotide	Sequence[1,e]	Molar yield[2]	RNase T_1 products	^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	RNase T_2 products	RNase T_1 products
P12	(3) GU[U] GU[G] (2) GU[A] (2) GU[C]	7.4	1 U 1.1 G	1.1		1.7		11.0	1 U* 2.6 G*	1.8	U*	
P13	AAGC[U]	0.9	1 C 0.9 AAG	0.9	A*	1.1	A*	0.8		0.9		AAG*
P14	GAAC[G]	0.9	1 AAC 0.7 G	0.8	C*	1.9	1 A* 1.1 G*	absent		0.8	A*	
P15	AAU[U] AAU[A]	1.9	AAU	absent		3.1	1 U* 2.1 A*	3.1	1 U* 2.0 A*	absent		
P16	AGC[A] AGC[C]	1.7	1 C 1.0 AG	2.0	A*	0.9		absent		2.8		1 C* 1.9 AG*
P17	GAC[U]	0.9	1 AC 0.9 G	absent		1.2	G*	0.9		1.0	A*	

Table A2 (continued)

		Uniform label		GTP-label[9]		ATP-label		UTP-label[9]		CTP-label[9]	
Oligo-nucleotide	Sequence[1,e]	Molar yield[2]	RNase T_1 products	^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	RNase T_2 products
P18	(2)AU[A] (3)AU[G]	6.0	AU	2.4		2.0	U*	5.4	1 A* 0.1 U*	absent	
P19	(2)GC[U] (1)GC[G]	3.3	1 C 0.9 G	0.3	C*	absent		1.6		3.8	1 G* 0.2 C*
P20	AAC[G]	1.3	AAC	1.0	C*	1.2	1 A* 0.1 C*	absent		1.1	A*
P21	G[C]	1.0	G	absent		0.1		0.2		0.3	
P22	(3)AC[A] AC[U]	4.9	AC	absent		3.0		1.5		5.4	A*
P23	(4)C[G] (4)C[A] (3)C[C]	13.8	C	3.4		3.4		–[8]		2.1	
P24	(6)U[G] (4)U[A] (8)U[C] (5)U[U]	24.8	U	5.9		4.2		4.6		7.2	

Footnotes to Table A1 and Table A2

(1) The following nearest neighbor nucleotides of each oligonucleotide are written in brackets.

(2) The molar yield was determined as follows: The sum of the radioactivity present in all the spots was divided by the total number of nucleotides present in the intact RNA fragment, which gives the average radioactivity per nucleotide. The radioactivity per nucleotide of each spot was divided by the average radioactivity per nucleotide to give the molar yield of the corresponding oligonucleotide.

(3) The number of ^{32}P-equivalents was determined as follows: The sum of the radioactivity present in all the spots was divided by the total number of ^{32}P-equivalents present in the intact RNA fragment to yield the average radioactivity per ^{32}P-equivalent. The radioactivity present in each oligonucleotide was divided by the average radioactivity per ^{32}P-equivalent to give the molar yield of ^{32}P-equivalents of the corresponding oligonucleotide.

(4) Not all the products were analysed, because of their low molar yield.

(5) The oligonucleotide is RNase T_1 labile and is easily overdigested to CUUUAA (T8, Fig.A-1 and A-2) +CG (T37).

(6) The oligonucleotide is RNase T_1 labile and easily overdigested to AA (T38, Fig.A-1) +CG (T37).

(7) The oligonucleotide is variable in its quantitation due to overdigestion products of T6 and T32 (see footnotes(5) and (6)).

(8) In the UTP-label this RNase A product was lost.

(9) The RNA fragments M-2a and M-2b were not resolved in these products.

(a), (b) The spots were not clearly resolved, which rendered their quantitation difficult.

(c) The quantitation was done by visual estimation of the darking of the autoradiogram.

(e) Expected values of molar yields are given in parenthesis.

(f) In addition to the analysis described, this oligonucleotide was identified as A_5U by electrophoresis on DEAE-paper at pH 3.5, using a mixture of oligonucleotides containing AU, AAU, AAAU, AAAAU and AAAAAU as reference markers. The unknown oligonucleotide coelectrophoresed with the standard AAAAAU (H. Weber, personal communication).

Table A3 Oligonucleotides obtained from partial digestion products of M-2a and M-2b, after complete nuclease digestion

Partial digestion product: CM-Pa (12.2)

T_1 oligonucleotides	Molar yield*)	Panc oligonucleotides	Molar yield*)
AAAAAUAG[C]	0.6	AGAAAAAU[A]	1.0
CCAAG[C]	1.0	AAGC[U]	0.9
AG[G/A/U]	1.8	AAU[A/U]	1.0
(C,U,AAU)	1.2	AGC[A/C]	1.0
		C[G/A/U/C]	0.9
		U[G/A/U/C]	1.0

Partial digestion product: CM-Pa (11)

T_1 oligonucleotides	Molar yield*)	Panc oligonucleotides	Molar yield*)
UUCCG[U]	1.0	G(AG,G)GU[A]	1.1
UUG[A/C]	1.0	AG(G,AG)AU[G]	1.0
AUG[U]	1.0	GU[G/A/U/C]	3.0
UCCG[A/U]	1.0	C[G/A/U/C]	3.1
AG[G/A/U]	3.0	U[G/A/U/C]	1.9
G[G/A/U/C]	3.2		
U	0.8		

Table A3 (cont.)

Partial digestion product: b(P/P)

T_1 oligonucleotides	Molar yield*)	Panc oligonucleotides	Molar yield*)
$AU_3CAAU_3CUCACUG[G]$	1.0	GAGGAU[U]	1.2
ACUACAG[U]	0.9	AGU[G]	1.1
UG[A/C]	1.2	AAU[A/U]	1.1
AG[G/A/U]	1.0	AC[A/U]	2.9
G[G/A/U/C]	1.0	C[G/A/U/C]	2.5
		U[G/A/U/C]	7.5
		G[G/A/U/C]	0.9

Partial digestion product: d (P/P)

T_1 oligonucleotides	Molar yield*)	Panc oligonucleotides	Molar yield*)
UAUACACAUG[G]	0.9	GAGU[C]	1.0
UCAUG[U]	1.1	GGC[A/U]	1.0
$(U,C_2)G[U/A]$	1.0	GU[G/A/U/C]	2.1
UAG[A]	0.9	AU[G/A]	3.2
AG[G/A/U]	1.1	AC[A/U]	1.8
CG[U/A]	1.2	C[G/A/U/C]	2.5
G[G/A/U/C]	1.0	U[G/A/U/C]	1.0
		AG	1.0

Table A3 (cont.)

Partial digestion product: 12 (P/P)

T_1 oligonucleotides	Molar yield*)	Panc oligonucleotides	Molar yield*)
$AU_3CAAU_3CUCACUG[G]$	0.9	GAGGAU[U]	1.1
UAUACACAUG[G]	0.9	GAGU[C]	1.0
UCAUG[U]	1.0	GGGC[G]	0.9
ACUACAG[U]	1.0	AGU[G]	1.0
$(U,C_2)G[U/A]$	1.0	GGC[A/U]	1.0
UAG[A]	1.1	GU[G/A/U/C]	2.0
UG[A/C]	1.1	AAU[A/U]	1.1
AG[G/A/U]	2.2	AU[G/A]	3.3
CG[U/A]	1.1	AC[A/U]	5.0
G[G/A/U/C]	4.0	C[G/A/U/C]	4.0
		U[G/A/U/C]	7.4
		AG	0.9

Partial digestion product: CM-Pa (7)

T_1 oligonucleotides	Molar yield*)	Panc oligonucleotides	Molar yield*)
$AU_3CAAU_3CUCACUG[G]$	0.7	GAGGAU[U]	1.0
UCAUG[U]	1.2	GAGU[C]	0.9
ACUACAG[U]	0.7	GGGC[G]	0.8
$(U,C_2)G[U/A]$	1.6	AGGC[C]	0.9
UG[A/C]	1.4	AGU[G]	0.8
AG[G/A/U]	3.7	GU[G/A/U/C]	2.1
CG[A/U]	1.2	AAU[A/U]	1.0
G[G/A/U/C]	5.0	GAC[U]	0.9
U	1.0	AU[A/G]	1.8
		AC[A/U]	2.1
		C[G/A/U/C]	3.6
		U[G/A/U/C]	8.0

Table A3 (cont.)

Partial digestion product: T (23)

T_1 oligonucleotides	Molar yield*)	Panc oligonucleotides	Molar yield*)
CUAAUAG[G]	1.0	AG(G,AG)AU[G]	0.9
AAAAAUAG[C]	0.9	AAGC[U]	0.9
AUG[U]	1.1	AAU[A/U]	1.0
CCAAG[C]	0.9	AGC[A/C]	1.0
AG[G/A/U]	1.6	C[G/A/U/C]	1.0
G[G/A/U/C]	1.1	U[G/A/U/C]	1.9
		AAAAAU	0.9
		G[G/A/U/C]	0.7

Partial digestion product: 12 (GTP-label)

T_1 oligonucleotides	No of ^{32}P-equiv.3)	Panc oligonucleotides	No of ^{32}P-equiv.3)
CUAAUAG[G]**)	2.5	G(AG,G)GU[A]	2.8
AAAAAUAG[C]	0.9	AG(G,AG)AU[G]	4.6
UUCCG[U]	1.0	AAGC[U]	1.1
UAUG[U]	0.9	AGC[A/C]	1.1
UUG[A/C]	2.2	AU[G]	1.0
AUG[U]	1.1	GC[G]	1.0
(U,C_2)G[A/U]	1.2	C[G]	2.3
CCAAG[C]	1.0	U[G]	3.0
AG	3.5		
CG[U/A]	0.9		
G	1.0		
CCG[A]	0.7		

Table A3 (cont.)

Partial digestion product: T (7)

T_1 oligonucleotides	Molar yield*)	Panc oligonucleotides	Molar yield*)
$AU_3CAAU_3CUCACUG[G]$	1.0	GAGGAU[U]	0.9
UCUCUAUAG[G]	1.2	GAGU[C]	1.3
UAUACACAUG[G]	0.9	GGGC[G]	1.0
UCAUG[U]	1.0	AGGC[C]	0.8
ACUACAG[U]	0.9	AGU[G]	1.1
$(U,C_2)G[U/A]$	3.1	GGC[A/U]	1.0
UAG[A]	1.0	GU[G/A/U/C]	3.5
UG[C/A]	1.0	AAU[A/U]	1.1
AACG[C]	0.5	GAC[U]	0.9
AG[G/A/U]	2.2	AU[A/G]	3.9
CG[U/A]	1.0	GC[G/U]	0.9
G[G/A/U/C]	5.0	AAC[G]	1.0
		AC[A/U]	5.0
		C[G/A/U/C]	7.3
		U[G/A/U/C]	10.6
		AG	1.0

Partial digestion product: T (32)

T_1 oligonucleotides	Molar yield*)	Panc oligonucleotides	Molar yield*)
UUCCUG[G]	1.1	AGAGGC[U]	1.2
CUG[A]	1.1	GGC[A/U]	1.0
CAG[A/C]	1.8	AGC[A/C]	1.0
AG[G/A/U]	1.1	C[G/A/U/C]	1.3
G[G/A/U/C]	1.5	U[G/A/U/C]	3.4
		G	1.0

Table A3 (cont.)

Partial digestion product: T (4)

T_1 oligonucleotides	Molar yield*)	Panc oligonucleotides	Molar yield*)
$AU_3CAAU_3CUCACUG[G]$	1.0	GAGGAU[U]	1.1
UCUCUAUAG[G]	0.9	GAGU[C]	1.2
UAUACACAUG[G]	0.9	GGGC[G]	0.9
CUUUAACG[A]	0.8	GAAU[G]	0.9
UCAUG[U]	1.2	AGGC[C]	1.3
ACUACAG[U]	0.9	AGU[G]	1.0
AAUG[U]	1.0	GGC[A/U]	0.8
$(U,C_2)G[A/U]$	3.1	GU[G/A/U/C]	4.4
UAG[A]	1.0	GAAC[G]	1.0
CUG[A]	0.9	AAU[A/U]	1.0
UG[A/C]	2.3	GAC[U]	1.2
AACG[C]	0.6	AU[A/G]	4.6
AG[G/A/U]	3.4	GC[U/G]	2.2
CG[U/A]	1.6	AAC[G]	1.2
G[G/A/U/C]	6.0	AC[A/U]	4.3
		C[G/A/U/C]	5.6
		U[G/A/U/C]	15.2
		AG	1.0

Table A3 (cont.)

Partial digestion product: 1 (P/P)

T_1 oligonucleotides	Molar yield*)	Panc oligonucleotides	Molar yield*)
$AU_3CAAU_3CUCACUG[G]$	1.0	GAGGAU[U]	1.1
UCUCUAUAG[G]	1.0	GAGU[C]	1.0
UAUACACAUG[G]	0.9	GGGC[G]	0.8
CUUUAACG[A]	1.0	AGGC[C]	1.0
UCAUG[U]	1.4	AGU[G]	1.2
ACUACAG[U]	0.8	GGC[A/U]	1.0
$(U,C_2)G[A/U]$	3.2	GU[G/A/U/C]	3.3
UAG[A]	1.2	GAAC[G]	1.1
UG[A/C]	1.2	AAU[A/U]	1.2
AACG[C]	0.5	GAC[U]	1.0
AG[G/A/U]	2.2	AU[G/A/U/C]	4.6
CG[U/A]	1.5	GC[G/U]	1.3
G[G/A/U/C]	4.5	AAC[G]	1.0
		AC[A/U]	3.7
		C[G/A/U/C]	7.5
		U[G/A/U/C]	14.6
		AG	1.0

Table A3 (cont.)

Partial digestion product: T2d (ATP-label)

T_1 oligonucleotides	No of ^{32}P-equiv.[3)]	Panc oligonucleotides	No of ^{32}P-equiv.[3)]
$AU_3CAAU_3CUCACUG[G]$	0.9	GAGGAU[U]	1.1
UCUCUAUAG[G]	1.2	AG(G,AG)AU[G]	1.0
UAUACACAUG[G]	0.9	AGAAAAAU[A]	0.9
CUUUAACG[A]	0.9	GAGU[C]	1.1
CUAAUAG[G]	1.0	GU[A]	2.0
AAAAAUAG[C]	0.8	AAGC[U]	0.9
UCAUG[U]	1.1	GAAC[G]	1.0
UUG[A]	1.1	AAU[U]	1.0
ACUACAG[U]	0.9	AAU[A]	1.1
$(U,C_2)G[A]$	1.1	GAC[U]	1.1
UAG[A]	1.0	AU[A]	1.9
UG[A]	1.3	AAC[G]	1.2
CCAAG[C]	1.0	G[A]	0.8
AACG[C]	0.6	AC[A]	3.3
AG[A]	1.3	C[A]	3.6
CG[A]	1.3	U[A]	4.1
G[A]	2.5		

Footnotes to Table A3

*)The molar yield was determined as described in the legend to Table A1 and A2, except that spots present in less than 0.4 molar yield were omited.

**)The oligonucleotide is a mixture containing the indicated oligonucleotide and T9a: UCACUG[C] (see Fig. A2).

3)The number of ^{32}P equivalents was determined as described in the legend to Table A1 and A2.

Table A4 Characterisation of the T_1 oligonucleotides T37a and T9a present in M-2b and absent in M-2a.

Products of T_1 oligonucleotides derived from homogeneously labeled RNA.

Oligonucleotide	Sequence[1]	Molar yield[2]	RNase A products
T9a	UCACUG [C]	1.0	G
			AC
			C
			2 U

Products of T_1 oligonucleotides from RNA synthesized in vitro in presence of one $-^{32}P$-nucleoside triphosphate.

GTP-label		ATP-label		CTP-label[9]		UTP-label[9]
^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	RNase A products	not analysed
0.7	U*	0.8	C*	1.5	U*	
					A*	
					G*	

Table A4 (continued)

Products of T_1 oligonucleotides derived from homogeneously labeled RNA.

Oligonucleotide	Sequence[1]	Molar yield[2]	RNase T_2 products
T37a	CCG[A]	1.0	G
		2.0	C

Products of T_1 oligonucleotides from RNA synthesized in vitro in presence of one α-^{32}P-nucleoside triphosphate.

GTP-label		ATP-label	CTP-label[9]	UTP-label[9]
^{32}P equiv.[3]	RNase T_2 products	^{32}P equiv.[3]	^{32}P equiv.[3]	^{32}P equiv.[3]
0.6	C*	1.0	0.5	0.1

[1,2,3,9] see legend to table A1 and A2

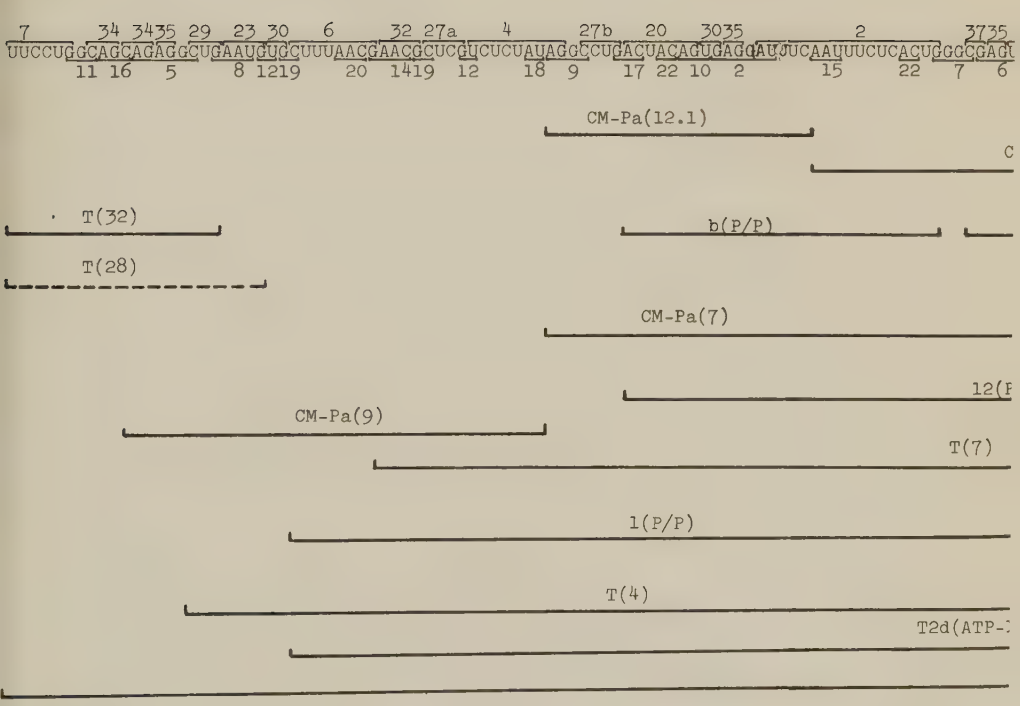
Table A5 Molar yields[2] of the RNase A products P12, P19, P21, P22, P23 and P24 of the RNA fragments M-2a and M-2b.

^{32}P-labeled RNA used	P12 GU		P19 GC		P21 G		P22 AC		P23 C		P24 U	
	M2a	M2b	M2a	M2b	M2a	M2b	M2a	M2b	M2a	M2b	M2a	M2b
Uniform label	8.1	9.4	2.3	4.0	1.0	0.5	4.0	5.o	12.2	13.3	20.4	22.2
GTP-label	1.1	1.1	0.3	0.6	abs.	abs.	abs.	0.2	3.4	4.2	4.9	6.0
ATP-label	1.8	2.2	abs.	0.2	0.1	0.5	3.2	3.4	3.5	4.4	4.3	4.8
CTP-label[9]	2.1		4.3		0.4		6.1		2.4		8.1	
UTP-label[9]	11.2		1.7		0.2		1.5		not analyzed		4.8	

[2,9] see legend to table A1 and A2

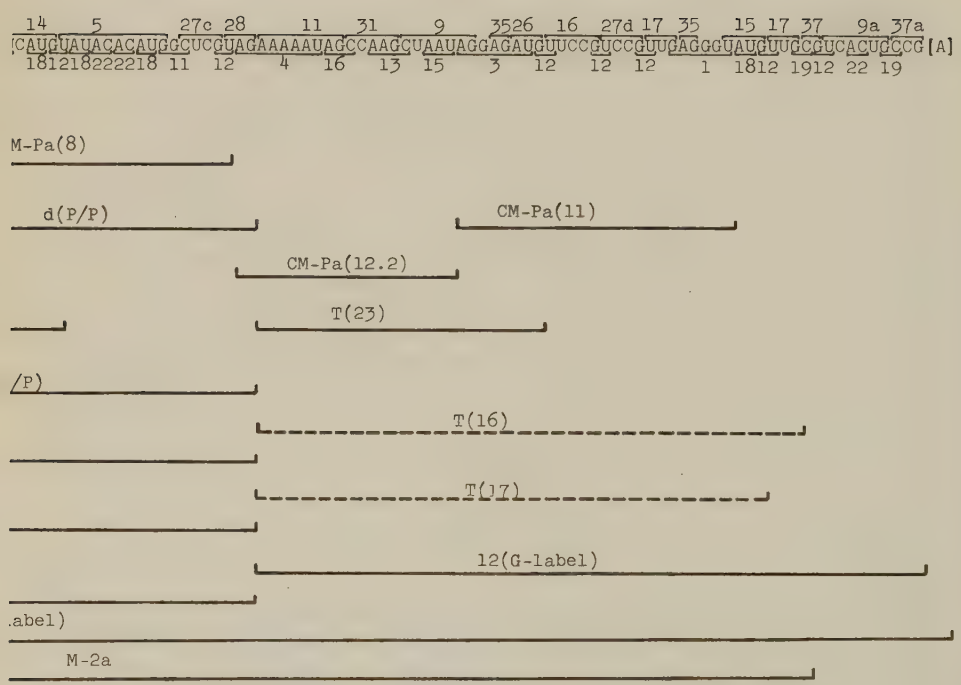
Figure A5

Complete nucleotid



The partial digest
lines. Other parti
broken lines. The
to the numbered RN
Mononucleotides ar

e sequence of the RNA fragment M-2b



.on products described in Table A3 are indicated by solid
ıl digestion products not described in detail are drawn in
ıumbers above and below the nucleotide sequence correspond
se T_1 and RNase A digestion products of figures A_{1-4}.
not numbered.

Curriculum vitae

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1954-1961	Primarschule in Hollenfels (Luxembourg)
1961-1968	Lycée de garçons de Luxembourg; Abschluss mit Certificat de fin d'études secondaires
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